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Our purpose is to explore the possibility of employing immunological intervention to hamper the carcinogenic process and the growth of spontaneous mammary carcinomas in mice. The final aim is to get enough information to decide whether a similar approach may be applied in humans at risk. The mammary glands of female Balb/c and FVB mice transgenic for the activated or amplified rat Her2/neu oncogene progress to carcinomas. We were able to significantly delay this carcinogenic process using repeated administrations of interleukin 12, which elicited the immune response of the host and inhibited tumor growth through the induction of antiangiogenic chemokines. An almost complete inhibition of tumorigenesis was obtained using a combination of IL-12 and of a vaccine containing allogeneic neu-postive tumor cells. The results support the idea that antigen specific and nonspecific signals are required to elicit an immune response that can protect the host from an ongoing carcinogenic process.

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N/A For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

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5. INTRODUCTION

Immunotherapy is emerging as an effective way to cure cancer (1-4) thanks to the dramatic progress that has led to the molecular and genetic definition of the tumor-host immune relationship. A detailed characterization of many tumor cell surface molecules that act as tumor associated antigens (TAA)(3) is now available (5-6). A second cornerstone has been provided by elucidation of the way in which TAA peptides are presented to T lymphocytes in association with glycoproteins of the major histocompatibility complex (MHC) (7-8), and the role of dendritic cells (9) and costimulatory (10), danger (11), and cytokine (12) signals. Genetic engineering of antibody molecules (13), soluble costimulatory signals (14-16) and tumor (17) and dendritic (18) cells is used to intensify the immune response and skew it towards Th1 or Th2 reactivity. This crucial information forms the groundwork for most ongoing immunotherapy clinical trials whose clinical setting is elicitation of an immune response in a tumor-bearing patient.

Determination of which kind of patients are eligible for phase III clinical trials is not a minor issue (19, 20). Practical and ethical constraints result in the enrolment of advanced cancer patients in phase I trials, whereas experimental mouse data suggest that the immunity induced by specific vaccination is much more effective in the inhibition of incipient than in the cure of established tumors. Elicitation of a significant response in animals with advanced tumors is exceedingly difficult and only a minority of tumor-bearing mice are cured (21). As a tumor increases in size, it becomes refractory to immunotherapy. Its genetic instability leads to the selection of antigenic variant clones (22, 23), while the characteristics of its stroma (24), the peculiarity of its blood vessels (25) and its release of immunosuppressive factors (26) build up a sort of privileged site proof against immune attack.

A similar picture is emerging from phase I immunotherapy trials. Only a few patients with established tumors display objective and in any event temporary responses (2, 3). The immunological performance status of the patients enrolled is obviously suboptimal. Most have already been treated in various ways and no longer respond to conventional therapy. Their tumor cells are selected because of their ability to escape immune reactions and their tumor masses can suppress an immune attack (21).

The lethality of a tumor, however, usually stems from the relatively small number of its cells that remain after its surgical excision and are not killed by radiotherapy and chemotherapy.

The importance of this issue lies in the experimental demonstration that active immunotherapy is effective against minimal residual disease and incipient metastases and in the control of tumor recurrences (27). Early immunotherapy after a successful conventional treatment is warranted. Clinical trials suggest that patients with minimal residual disease or expected to present recurrences after a long interval are those for whom immunotherapy may prove really effective, since it induces a prolonged tumor free-survival if not a complete cure (1, 4). Cancer vaccines tested as single agents in advanced melanoma patients are being tested in apparently disease-free patients in combination with chemotherapy. Significant results are expected from this more rational clinical approach (28). Once the efficacy of therapeutic immunization is demonstrated, it may also be proposed as an at-home or day-hospital method for the elicitation of a long-lasting immunity following the conventional management of a small tumor (17).

If immunotherapy is most effective in the early stages of tumor growth, consideration can be given to an even more radical view. The use of immunological measures to prevent or forestall cancer in healthy persons has not received much attention. This is surprising since most of the experimental data obtained with transplantable tumors show that new vaccines preimmunize mice against even a poorly or apparently non-immunogenic tumor challenge (12, 29). Furthermore, the nonspecific immunity elicited by local and systemic cytokines effectively inhibits incipient tumors until they overcome a critical threshold and become clinically evident (27). Numerous and unambiguous experimental data show that the efficacy of both nonspecific and specific immunity declines as a tumor progresses (21, 27). Whether willfully or unthinkingly, however, the evidence from preimmunization-tumor challenge experiments and the cytokine-induced collapse of incipient tumors is strained to demonstrate the efficacy of immunological measures in tumor therapy and not accepted for what it really says (30), namely that immune reactivity possesses a great preventive potential, whereas its real therapeutic efficacy against established tumors is altogether another question (17).

Immunoprevention of cancer would have many advantages on its side. Healthy subjects, for example, may be expected to mount a more effective and powerful response than patients who have already been treated in various ways, while if the target tissue is still normal or displays no more than a localized preneoplastic lesion, the chances of success should be greater than when dealing with unresectable or disseminated tumors (31). Preneoplastic lesions do not yet display genetic instability, TAA mutations and selection of the TAA negative clones that characterize established tumors. They should also be more permeable to immune mechanisms,

since their cells do not markedly remodel the extracellular matrix nor produce suppressive factors, and their vessel endothelium is not yet refractory to leukocyte extravasation (32-34). Several mutations in oncogene products are required for transformation. By contrast, an alerted immune patrol would detect the initial mutations and be ready to intervene before complete transformation takes place. Although antigen(s) associated to preneoplastic lesions (as well as those for many established neoplasms) have not been yet identified, the product of mutated oncogenes are probable candidates (34, 36).

NONSPECIFIC IMMUNITY IN CANCER PREVENTION

The selection of not-yet patients and healthy individuals eligible for immunoprevention depends on the kind of treatment envisaged. Enhancement of nonspecific immunity and specific antitumor vaccination are two possible approaches. The advantage of a nonspecific antitumor response is that it can be directly applied to a broad range of individuals, irrespective of the type of TAA their foreseeable tumors may eventually express. However, it is unfeasible to imagine healthy persons being nonspecifically treated for long periods. The results of the mouse experiments indicate that nonspecific stimulation should thus be restricted to non-yet patients with a genetic risk of cancer (34), individuals exposed to high carcinogen doses (37), patients with a preneoplastic lesion and those that probably have minimal residual disease after a successful conventional treatment (27). Many not-yet patients with a high risk of cancer are, in fact, being recruited in ongoing programs to screen for preneoplastic lesions or gene mutations that predispose to cancer.

Women at risk for breast cancer or with preneoplastic lesions form a category for which nonspecific immunoprevention could be considered as a practical option.

However, the disclosure of a genetic risk of cancer and the presence of a preneoplastic lesion raise complex issues (38). Not a few individuals will find it difficult to cope with this information and may become deeply anxious about the possibility that they may have a cancer. Routine cancer screenings, prophylactic mastectomy and chemoprevention are all unpleasant and additionally stressful options.

But what has nonspecific stimulation of immune reactivity to offer? A study of immunosurveillance against preneoplastic skin carcinomas suggested that it is not selective, since their elimination was in no way related to their degree of malignancy. The extent to which nonspecific stimulation can prevent the onset of cancer in cases where a risk exists has been investigated by Noguchi et al. (37) in Dr. L. J. Old's laboratory. In their experiments, tumors

were induced in BALB/c mice by subcutaneous injection of 3-methylcholanthrene. Delayed tumor appearance and reduced incidence were observed in mice receiving 100 ng systemic interleukin- (IL-) 12 five days a week for 18 weeks (3 weeks on and 1 week off) during tumor latency. Secondary interferon-γ, interleukin-10, and tumor necrosis factor-α were evident in their sera. A high production of interferon-γ by CD8 T cells and a Th2 -> Th1 or Th0 shift in the cytokine secretion profile of CD4 T cells were also noted.

SPECIFIC IMMUNITY IN CANCER PREVENTION.

Specific vaccination of persons at risk and healthy individuals constitutes a very different scenario. Characterization of specific gene alterations or detection of preneoplastic lesions may indicate which organ and tissue are at risk. In a few cases, more precise information may show which oncogene product will probably be overexpressed or expressed in an altered form and allow vaccination against a single, specific TAA. Molecular characterization of altered gene products predictably destined to become TAA will be the first step towards the engineering of selective vaccines. Otherwise, the patient should be vaccinated against the TAA most commonly expressed by the tumors foreseeable in a given organ.

Many new antitumor vaccines that induce an effective resistance to subsequent tumor challenge and inhibit minimal residual disease are already available (12, 29). The question whether specific immunization can be successful once a cell population has been subjected to the initial carcinogenic hit has rarely been examined experimentally. It can, however, be plausibly suggested that cytokines and more conventional adjuvants could induce an effective immune response against ignored or fully tolerated antigens. The specific immunity elicited in mice transgenic for rat Her-2/neu is a sign that specific vaccination induces strong immune responses against such antigens and may thus inhibit oncogenesis and extend survival.

One can also envisage the even wider application of antitumor vaccines to prevent tumors in the general population as is done for infectious diseases.

As many TAA are shared by a variety of tumors, preventive immunization against most common human cancers with not many more than twenty TAA would seem conceivable. A possible list would include the infectious agents mentioned earlier, mutated oncogenes, telomerase catalytic subunit and antigen of the MAGE family. The erratic boundary between tumor immunity and autoimmunity, however, means that the risk of inducing an autoimmune disease is a major concern. This risk would be much weightier in the vaccination of healthy

individuals as opposed to individuals at risk, where the scales of risk-benefit are markedly biased by the higher risk of cancer and the consequent shorter life expectancy.

The planning of vaccines "à la carte" by genetic engineering may be a way to selectively trigger reaction mechanisms which ignore cells that express a low density of the target antigens or are less prone to induce a widespread autoimmunity. Consideration must also be given to the balance between the kind of potential autoimmunity and the degree of lethality of the possible tumor. Experimental studies should address this issue in detail.

In conclusion, immunoprevention of cancer seems a distant, but plausible prospect. This research project address the experimental elucidation of a few of its critical issues. It could provide important information for its application in persons at risk.

6. BODY

TASK 1. PREVENTION OF MAMMARY CARCINOMA WITH RECOMBINANT IL-12.

The specific aim is to prevent mammary carcinoma in Balb-NeuT and FVB-NeuN female mice by repeated systemic administrations of IL-12.

The ability of IL-12 to prevent tumors when administered to mice with a genetic risk of cancer was therefore studied by us (34) in two lines of transgenic mice expressing the rat HER-2/neu oncogene under the transcriptional control of mouse mammary tumor virus (34). Female BALB/c (H-2d) carrying the transforming HER-2/neu oncogene (BALB-neuT) show no morphological abnormalities of the mammary gland until 3 wk of age. They then progress through atypical hyperplasia to in situ lobular carcinoma and at 33 wk of age all ten mammary glands display invasive carcinomas. In adult FVB-neuN mice (H-2q) carrying the HER-2/neu protooncogene, neoplastic progression is less impetuous, as shown by a longer latency (38-49 weeks) and a lower tumor multiplicity (mean of 2.6 tumors/mouse). Treatment with IL-12 (five daily i.p. injections, 1 week on, 3 weeks off; the first course with 50 ng IL-12/day, the following with 100 ng IL-12/day) begun at the 2nd week of age in BALB-neuT mice and at the 21st week in FVB-neuN mice markedly delayed tumor onset and reduced tumor multiplicity. In both lines, tumor inhibition was associated with deficient peri- and intra-tumoral angiogenesis, infiltration of reactive cells, production of pro-inflammatory cytokines, and inducible NO synthetase activation.

We therefore set out to define the progression stage in which these mechanisms are most effective. Should IL-12 administration be proposed as a preventive measure in not-yet patients only, or can it also be of benefit once overt preneoplastic lesions are diagnosed? This is a significant question since genetic screening programs are singling out healthy not-yet patients (38) and early diagnosis programs are detecting pre-neoplastic lesions. Technical and specific features related to this task of the program are reported in detail in ref. 34 and Appendices #1, #2, #3, and #5. Groups of BALB-neuT and FVB-neuN mice received IL-12 at progressive times during carcinogenesis.

IL-12 (Genetics Institute, Cambridge, MA) in Hank's balanced salt solution supplemented with 0.01% mouse serum albumin (MSA, Sigma, St. Louis, MO) was administered intraperitoneally. Mice received seven 5-day courses of MSA only (MSA controls) or MSA plus IL-12. Other groups of mice remained untreated. To evaluate the ability of IL-12 to inhibit this progression, mice received seven 5-day courses of IL-12 at different times. As Balb-NeuT mice already display hyperplasia of small lobular ducts and lobules at 3 weeks of age, "Chronic" IL-12 administration started in the 2nd week and continued until the 25th week. Both a delay in the onset of the first tumor and a 50% reduction of the number of mammary glands with a palpable tumor at 33 weeks when the experiment ended were observed as compared to MSA controls. To assess whether IL-12 is also effective during later phases, other mice were first treated at the 13th week of age, when hyperplasia takes the form of a carcinoma in situ. Courses continued until the 25th week. This "Late" treatment did not delay the onset of the first tumor, but none the less reduced the number of tumors at week 33 by 22%. The "Early" treatment began at the 2nd week and continued until week 14. The delay of first tumor onset and the reduction of the number of tumor are significantly higher than in "Chronic" treatment. When the "Early" treatment was further split into shorter four-week administration schedules, much less protection was observed.

The efficacy of IL-12 in Balb-NeuT mice suggests that evolution of the tumor:host angiogenic relationship, rather than intrinsic proliferative properties of transformed mammary cells is the point of no return for its activity. At least part of this antitumor activity appears to depend on its ability to inhibit the angiogenesis associated with mammary hyperplasia. Immunohistochemical staining with anti-CD31 monoclonal antibody shows that rich microvascularisation inside preneoplastic lesions corresponds to their progression towards carcinoma, as shown in other tumor systems. This progression phase appears to be particularly appropriate for an angiostatic intervention. Indeed, the most significant delay in tumor onset and

progression is observed with the "Early" treatment, which induced both a scanty vascularization and poorly developed hyperplastic foci.

The importance of the time of IL-12 administration was further assessed with FVB-NeuN mice, in whom an overexpressed Her-2/*neu* protooncogene induces mammary carcinomas after a markedly longer latency. The "6-week-old" treatment consists in a lifetime administration of IL-12 and is conceptually similar to the "Chronic" treatment of Balb-NeuT mice. While on the "22-week-old" treatment the first course was markedly delayed, it still started before an evident spreading of preneoplastic lesions. Both treatment schedules delay the onset of carcinomas and their multiplication. The period between the 22nd and the 28th week appears to be of critical importance, as the "28-week-old" protocol confers a negligible protection only. During these six weeks, in fact, normal mammary glands progress towards atypical hyperplasia and then to carcinoma in situ and invasive carcinoma.

The equivalent results from Balb-NeuT and FVB-NeuN mice suggest that IL-12 effectively inhibits mammary carcinogenesis when its administration accompanies the angiogenic switch. Its anti-angiogenic effect appears to rest on the increased serum levels of IFN-γ and TNF-α released by activated T lymphocytes and NK cells, whose anti-angiogenic and angiotoxic activity is stronger on the fragile capillary sprouts that accompany the shift from the preneoplastic to the neoplastic condition. Downstream mediators elicited by IL-12 may also act on neoplastic cells in which they downregulate the production of pro-angiogenic molecules and upregulate the release of anti-angiogenic factors, such as IP-10 and MIG (27). Following the transition from hyperplasia to in situ and invasive carcinoma, capillary sprouting is restrained. The poor efficacy of late treatment may depend on the lower sensitivity to IL-12-induced angiostasis of the mature and differentiated blood vessels of the advanced neoplastic lesions. The decreased number of microvessels per microscopic field in both in situ and invasive carcinoma in comparison to hyperplastic areas suggests that this type of carcinoma once developed no longer requires a profuse vascular supply. The few vessels of the stroma of neoplastic lobular-alveolar structures are enough to sustain their relatively low rate of proliferation. By contrast, blood supply is a critical factor for most fast-growing transplantable tumors, even during their later stages. This necessity may account for IL-12's high efficacy against these tumors, even when they are large. With tumors that progress slowly, anti-angiogenic activity is only efficacious in specific progression stages. This narrow window of activity might account for the ineffectiveness

of IL-12 in the management of human cancer, since only patients bearing advanced tumors are enrolled in clinical trials.

The anti-tumor action of IL-12 is not confined to its indirect influence on endothelial cells. Directly or through secondary cytokines it triggers lytic activity and mediator release in a variety of tumor-infiltrating leukocytes, thus offsetting the continuous generation of new transformed cells (27). The efficacy of IL-12 probably rests on the sum of its activities, and not simply on blocking of tumor neoangiogenensis, important as this may well be. In effect, further subdivision of the "Early" protocol into shorter treatment periods markedly reduced IL-12 efficacy. The lower efficacy of "Chronic" versus "Early" treatment could indicate that continuous IL-12 administration is suppressive, though this possibility is not endorsed by the results in FVB-NeuN mice. It should be noted that from the second course mice of both strains received daily 100 ng/day IL-12 (i.e. around 4.5-7.7 μg/Kg). This dose is well tolerated and almost no-side effects appeared (34). It is probably close to the optimal active dose, since a ten or twenty-fold reduction abolishes its activity.

In conclusion, our data suggest that IL-12 effectively impairs the *neu* oncogene driven progression of mammary carcinogenesis by interfering with the passage from atypical hyperplasia to invasive carcinoma. This interference appears to mostly depend on indirect inhibition of tumor-associated angiogenesis. Its lower efficacy in more advanced lesions and the dose range required pose some constraints on the use of IL-12 as an immunological alternative to current management of manifest neoplastic lesions. Nevertheless, the efficacy of IL-12 points to enhancement of nonspecific immunity as an effective way to prevent mammary tumors in individuals at risk. Lifetime administration is not required for genetically determined cancers with a long natural history, whereas definition of the carcinogenic events may enable preventive

TASK 2-3. PROPHYLACTIC VACCINATION WITH CYTOKINE GENE-TRANSDUCED TUMOR CELLS.

The aim of this task is to apply to cancer immunoprevention cellular vaccines made of engineered tumor cells. To this end we established and characterized a number of new cell lines and clones derived from FVB-NeuN. A clone expressing high levels of neu (named N202.1A, see appendix #4) was used as recipient for transduction with genes coding for various cytokines (IFN-γ, IL-2, IL-12, IL-15). Previous studies by our group have shown that allogeneic histocompatibility molecules (MHC class I) can significantly increase the immunogenicity of

cellular vaccines engineered with cytokine genes, thus we chose to test the efficacy of these cells of H-2^q haplotype in allogeneic Balb-NeuT (H-2d) transgenic mice, the most aggressive model available.

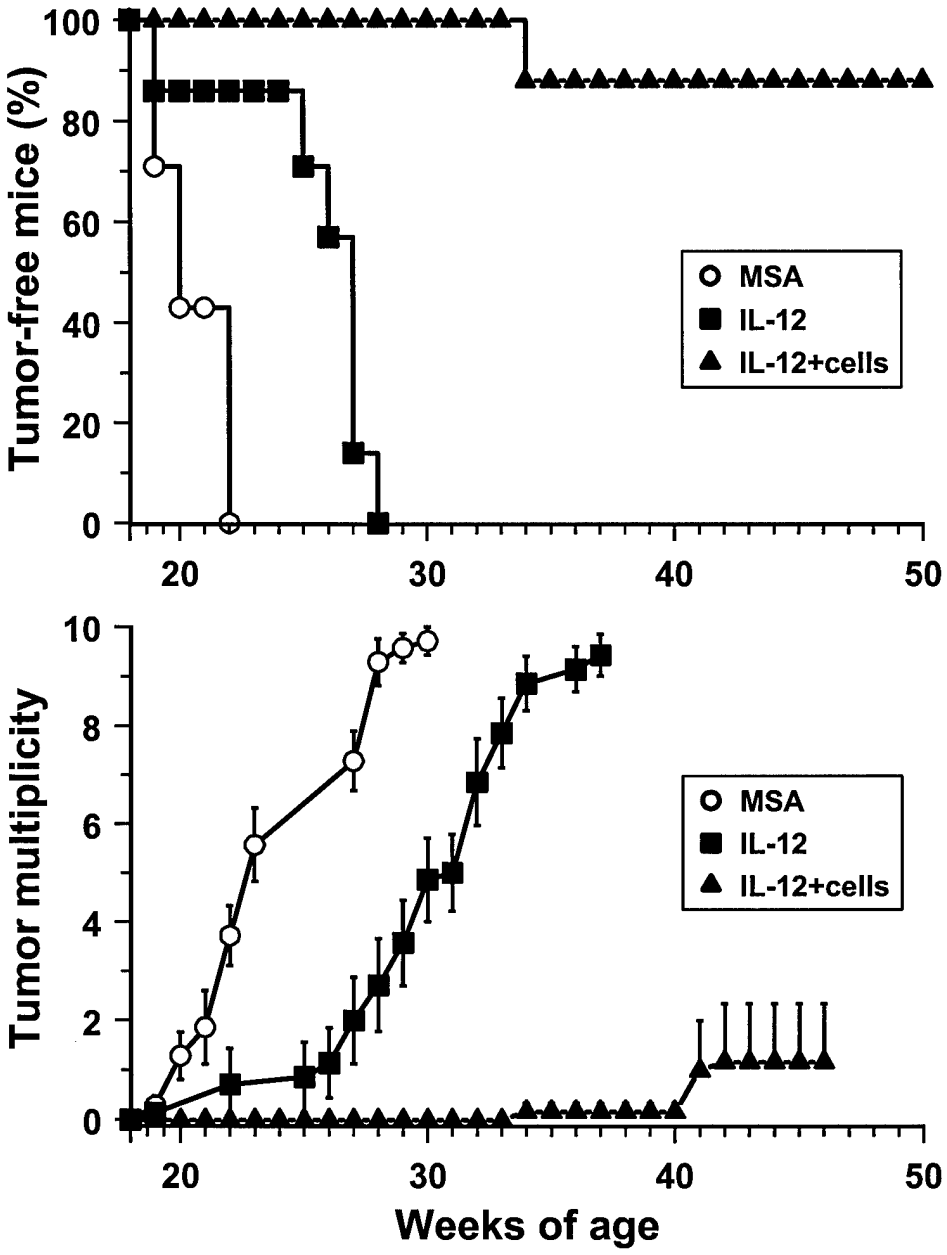
Mice were vaccinated every 28 days (twice/week for two weeks) from the 6^{th} week of age with transduced cells. Preliminary data show that cells transduced with the IL-12 genes provided the best protection from tumor development (Table 1).

Table 1. Prevention of mammary carcinoma by vaccination with cytokine gene transduced cells.

Cell vaccine	**Gene transfection**	**Tumor-free mice at 30 weeks of age**	**%**
None	None	0/8	0%
N202.1A	None	0/7	0%
N202.1A	IFN-γ	4/8	50%
N202.1A	IL-2	3/8	38%
N202.1A	IL-12	8/8	100%
N202.1A	IL-15	2/8	25%

To further increase the efficacy, cellular vaccines were combined with the chronic IL-12 treatment of mice outlined under Task 1. We designed a multicomponent vaccination strategy based on alternating administrations of allogeneic trasngenic tumor cells and of IL-12. The first cellular vaccine tested was based on IFN-γ transfected cells. In summary we added together four different signals: $p185^{neu}$, IFN-γ, allogeneic MHC class I antigens, and IL-12. The onset of mammary carcinomas was strongly inhibited in transgenic mice receiving this type of treatment (Figure 1), and 88% of vaccinated mice are alive and tumor-free at 50 weeks of age, as opposed to 0% of MSA (murine serum albumin) treated controls. This is the most effective type of tumor prevention demonstrated so far in Balb-NeuT mice.

Figure 1. Comparison of IL-12 alone and IL-12 + cellular vaccine in the protection of Balb-NeuT mice from the development of mammary carcinoma.



We dissecteed the elements required to produce a significant immunity from mammary carcinoma growth. Table 2 shows that both the specific vaccine and the nonspecific stimuli such as IL-12 and allogeneic MHC antigens were required to produce a maximal effect..

Table 2. Analysis of individual components of the multicomponent vaccine.

Cell vaccine			rIL12	Tumor-free mice at	
neu	allo-MHC	IFN-γ gene		24wk of age	46 wk of age
-	-	-	-	0%	0%
+	+	+	+	100%	88%
+	+	+	-	88%	12%
+	+	-	+	88%	88%
+	+	-	-	63%	12%
-	-	-	+	86%	0%
-	+	-	-	0%	0%

TASK 4. VACCINATION WITH HER-2/*NEU* PEPTIDES

The specific aim is to prevent mammary carcinoma by vaccination with peptides derived from the Her-2/*neu* oncogene. Since the binding motifs of the H-2^d haplotype are well known (while H-2^q has not been thoroughly studied) we have studied this approach in Balb-NeuT mice, of H-2^d background. The first step was to identify peptide sequences in the neu gene that bind to H-2^d class I gene products leading to receptor-mediated recognition by T lymphocytes. We derived the peptide motifs for binding in the grooves of H-$2D^d$, H-$2K^d$, H-$2L^d$. The chosen peptides derive all from the intracellular domain of the $p185^{neu}$. They are designated: P114-003 (amino acids 249-257), selected for binding to H-$2D^d$; P114-002 (amino acids 558-566), selected for binding to H-$2K^d$; and P114-004 (amino acids 66-76). This 11 amino acid long peptide binds to both H-$2L^d$ and H-$2D^d$. All the peptides were synthetized by Primm Srl (Milan, Italy).

The second step was to assess the immunogenicity of the three peptides and analyze the immune response by using short-term vaccination protocols and in vitro cell-mediated cytotoxicity assays. First, immunogenicity was evaluated in Balb/c mice (H-2^d), where the rat

$p185^{neu}$ protein is an exogenous antigen with marked homology with autologous mouse $p185^{neu}$ protein. Mice were immunized intradermally, once a week for 4 weeks, with 100 μg of peptide in 50 μl of PBS. One week after the last immunization, mice were challenged subcutaneously with a cell line derived from a Balb-NeuT carcinoma (TUBO cells) and observed for tumor appearance and progression. No differences were observed between control and peptide-vaccinated mice (data not shown). In other experiments, mice received 1 x 10^7 syngeneic spleen cells (Spc) pulsed overnight with peptides. One week after the last immunization, a few mice were sacrificed to asses Spc cytotoxity against TUBO cells after six days in vitro restimulation with mitomycin-C treated (Sigma, St. Louis, MO; 100 μg/10^7 cells/ml for 30 min) TUBO cells. Cytotoxic activity was expressed as Lytic $Units_{20}$ (LU). Other mice were challenged subcutaneously with TUBO cells and observed for tumor appearance and progression. Our data show that Spc from these vaccinated mice are able to prime for a TUBO specific cytotoxic response, as shown by in vitro cytotoxicity assays (Table 3).

Table 3. Cytotoxicity against TUBO cells of Spc from vaccinated mice after in vitro stimulation with Mitomycin-C treated TUBO cells.

Mice immunized with:	**Cytotoxicity (LU ± SD)**
None	42 ± 7
Spc not pulsed	193 ± 12
Spc pulsed with P114-002	246 ± 41
Spc pulsed with P114-003	302 ± 14
Spc pulsed with P114-004	227 ± 18

Despite this feeble reactivity, Spc from immunized mice do not inhibit the in vivo growth of TUBO cells (Table 4).

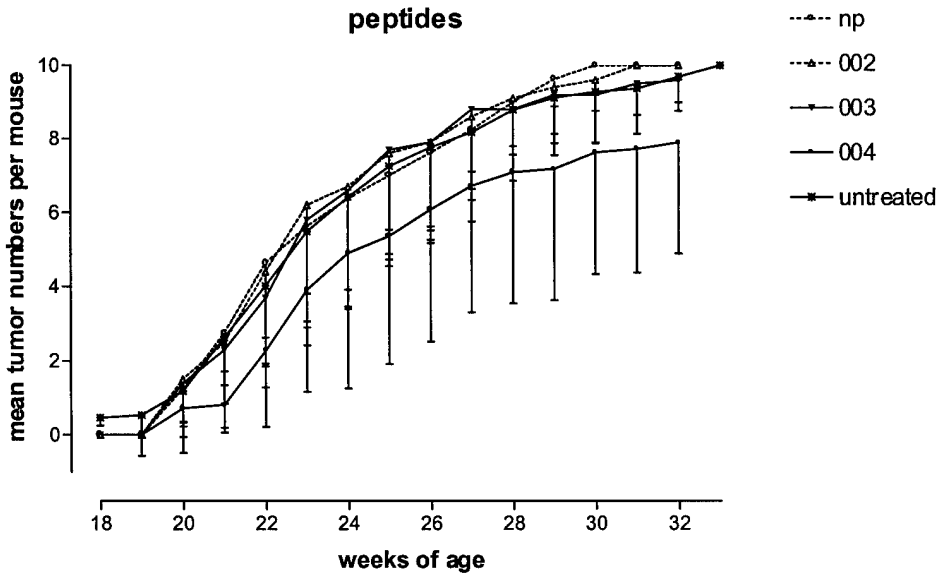
Table 4. Effect of vaccination with Spc pulsed with peptides on the in vivo growth of TUBO cells in Balb/c mice.

Mice immunized with:	Tumor take	Latency time[a]	Survival time[b]
None	5/5	24 ± 1	48 ± 8
Spc not pulsed	5/5	24 ± 2	49 ± 2
Spc pulsed with P114-002	5/5	26 ± 2	54 ± 7
Spc pulsed with P114-003	5/5	25 ± 1	51 ± 3
Spc pulsed with P114-004	5/5	26 ± 1	48 ± 9

[a]Latency time: time in days between the challenge and the appearance of tumors > 3 mm mean diameter.
[b]Survival time: time in days between the challenge and the appearance of tumors > 10 mm mean diameter.

The third step was the actual vaccination of Balb-NeuT mice in which rat $p185^{neu}$ is a fully tolerated self antigen. Starting from the seventh week of age, mice were immunized with 10^7 syngeneic Spc pulsed with peptides, once a week for four weeks, followed by three weeks off. This course was repeated four times. As shown in Figure 2, no significant differences were observed as far as the mean number of tumors per mouse was considered. However, mice vaccinated with Spc pulsed with P114-004 displayed lower values.

Figure 2. Effect of vaccination with Spc not pulsed (np) or pulsed with the various peptides (002, 003, 004) on the development of spontaneous mammary carcinomas in Balb-NeuT mice. Each group: 10 mice



TASK 5. STUDIES IN IMMUNODEPRESSED MICE

The specific aim of this task is to use immunodificient mice to define the immune mechanisms involved in the prevention of mammary carcinoma. This task will be the main focus of the third year of the project

7. CONCLUSIONS

Our results show that the carcinogenic process giving rise to mammary carcinoma can be prevented using different immunological approaches. The best results were obtained using a combination of specific and nonspecific stimuli that included a vacccine based on allogeneic tumor cells and recombinant IL-12. The application of similar approaches to human situations could lead to improvements in the prevention of mammary carcinoma.

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LABORATORY INVESTIGATION

Appendix #1

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Analysis of Mammary Carcinoma Onset and Progression in HER-2/*neu* Oncogene Transgenic Mice Reveals a Lobular Origin

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SUMMARY: Morphologic examinations of mammary neoplasias arising in BALB/c (H-2^d) mice carrying the activated rat HER-2/*neu* oncogene (BALB-NeuT), and in FVB (H-2^q) mice bearing the wild-type proto-oncogene (FVB-NeuN), indicate that both conditions result in a very human-like lobular carcinoma of alveolar type, whose histotype is the result of the preferential expression of HER-2/*neu* products in the epithelium of lobular ducts and lobules. Detailed analysis of tumor progression indicates that transition from lobular hyperplasia to overt carcinoma is associated with a high epithelial proliferation rate, as assessed by anti-proliferating cell nuclear antigen immunostaining, and coincides with the activation and maximal extension of tumor angiogenic process as assessed by microvessel count (anti-CD31), anti-β_3 integrin, and anti-laminin immunostaining. Neovascularization is accompanied by vascular endothelial cell growth factor and basic fibroblast growth factor production by hyperplastic epithelial cells. By contrast with the BALB-NeuT tumors, E-cadherin expression is almost nonexistent in those arising in FVB-NeuN mice and this may explain their high metastatic potential. Despite their different kinetics, however, the lung metastases observed in both strains are histologically similar and resemble the primary tumor. Both strains can thus be proposed as models for "in vivo" investigation of the origin and progression of the alveolar type of lobular mammary carcinoma and the testing of new therapeutic approaches. (*Lab Invest 1999, 79:1261–1269*).

Breast cancer is the most frequent malignancy of woman worldwide (Parkin et al, 1999). Rodent models have been particularly useful in illustrating its pathogenesis and evaluating its response to therapy (Anderson, 1992). These models, however, do not reflect the complex variety of human mammary cancer, because they are almost exclusively virus or chemically induced ductal adenocarcinomas (Russo and Russo, 1996). Generation of mouse strains transgenic for the HER-2/*neu* oncogene offers the opportunity to investigate a spontaneously arising mammary carcinoma and evaluate the "in vivo" role of HER-2/*neu* in cancerogenesis and progression (Bouchard et al, 1989; Guy et al, 1992, 1996; Lucchini et al, 1992; Muller et al, 1988; Suda et al, 1990).

The HER-2/*neu* oncogene is involved in human mammary cancerogenesis. Its amplification and overexpression, in fact, have been observed in a large percentage of primary human breast cancers and seem to be inversely correlated with survival (Di Giovanna et al, 1996; King et al, 1985; Slamon et al, 1987, 1989), though the significance of this correlation varies widely from one study to another.

Previous genetic, biochemical, and morphologic studies of HER-2/*neu* in mouse mammary carcinogenesis have provided a schematic representation of its contribution to tumor progression in both mice and humans (Di Giovanna et al, 1998). However closer histologic and pathologic investigation of HER-2/*neu*–associated tumor onset and progression is needed to determine the extent to which the mouse and human forms converge and diverge.

In this article we report that BALB/c transgenic female mice carrying the activated rat HER-2/*neu* oncogene (Boggio et al, 1998; Muller et al, 1988) quickly develop mammary tumors pathologically similar to those developed more slowly by transgenic FVB female mice carrying the wild-type proto-oncogene and overexpressing its product (Guy et al, 1992). Both

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tumors are similar to the alveolar-type human lobular mammary carcinoma.

Inclusion of a lobular type in the histologic classification of rodent mammary tumors (Russo and Russo, 1996) makes it more detailed and fully comparable to that of human forms (Rosai, 1996). Herein we propose these two strains of HER-2/*neu* transgenic mice as a model for investigation of the mechanisms underlying the origin and progression of lobular breast cancer.

Results

Histologic Examination of HER-2/neu Transgenic Mice Mammary Tissue

Female BALB/c (H-2^d) mice carrying the activated rat HER-2/*neu* oncogene (BALB-NeuT) showed no palpable lesions of the mammary gland until 15 weeks of age. They then began to develop multiple mammary tumors that progressively involved all 10 glands by the 33rd week. No appreciable differences in tumor natural history were observed in the HER-2/*neu* transgenic CD1 mice mated with BALB/c to obtain BALB-NeuT mice. Female FVB (H-2^q) mice carrying the HER-2/*neu* proto-oncogene with HER-2/*neu* product overexpression (FVB–NeuN) developed mammary carcinomas with a longer latency (38th to 49th week) and a lower multiplicity (mean of 2.6 tumors/mice).

Histologic examination of BALB-NeuT mammary tissue showed that widespread atypical hyperplasia of small lobular ducts and lobules was already evident at 3 weeks (Fig. 1a) and characterized by proliferation of a relatively uniform population of round epithelial cells assuming a stratified appearance with no formation of epithelial bridges.

Starting at the 11th week, the ductules and acini within the lobules were distended by the solid, occlusive growth of this epithelial cell population (Fig. 1b). The myoepithelial cell layer was scarcely represented or absent around the neoplastic lobular structures. The ductular and acinar outlines remained distinct and separate from one another, with persistence of intervening stroma. These features were distinctive of lobular carcinoma "in situ." At nearly the 20th week, alveolar groups of neoplastic cells with no myoepithelial lining infiltrated the surrounding adipose tissue (Fig. 1c). The linear, "Indian-file" arrangement of tumor cells and their circumferential growth around ducts and lobules ("targetoid growth") (Rosen and Oberman, 1993) were not observed. Histologic examination performed in transgenic CD1 mice revealed the development of mammary lobular carcinoma with morphologic features similar to those found in BALB-NeuT mice (data not shown). Thus the genetic background of BALB/c did not alter the carcinogenesis and the tumor phenotype in transgenic CD1 mice.

Histologic examination of FVB-NeuN mammary tissue revealed normal ductular and lobular structures until 35 to 37 weeks, after which foci of epithelial hyperplasia evolving to lobular carcinoma "in situ" and then to invasive lobular carcinoma of the alveolar type were found. The histologic features of this carcinoma were similar to those observed in BALB-NeuT mice, though the proliferating cell population displayed minor variations in size and in cytoplasm staining. Lung metastases recovered from both strains were histologically similar to the primary tumor.

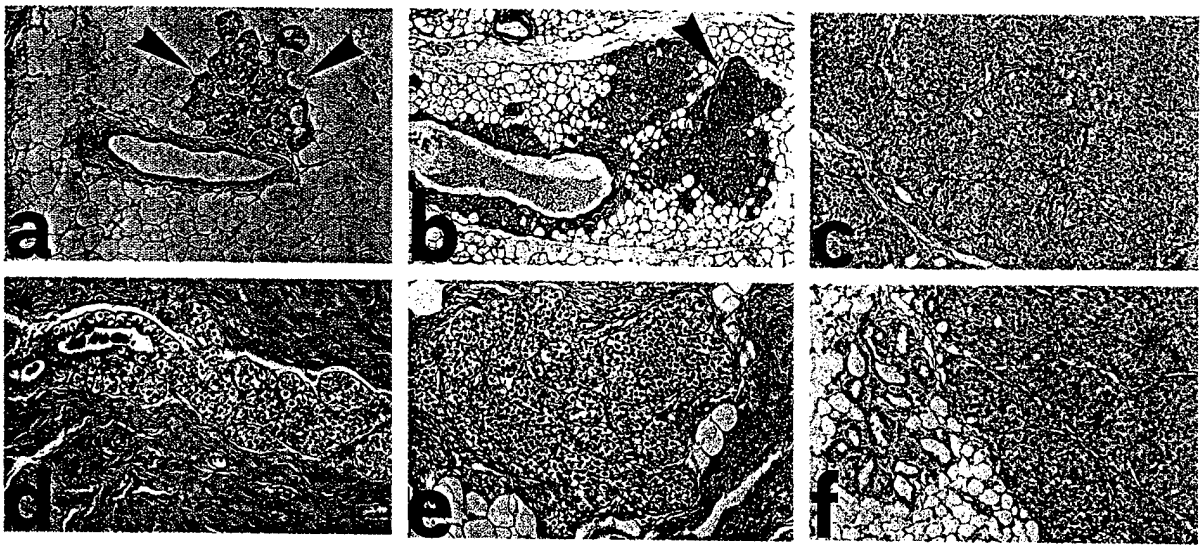

Figure 1.

Histologic features of lobular carcinoma development in rat HER-2/*neu* transgenic mice (a–d). In 5-week-old BALB-NeuT mice, small lobular ducts and lobules (*arrowheads*) are almost completely occupied by round epithelial cells assuming the stratified appearance of hyperplasia (a). In 13-week-old BALB-NeuT mice, the neoplastic epithelial cell proliferation assumed the solid occlusive intralobular growth typical of the lobular carcinoma in situ (*arrowhead*) (b). Typical pattern of lobular carcinoma with alveolar arrangement in BALB-NeuT (c) and in FVB-NeuN (f) mice. The histologic features of human mammary lobular atypical hyperplasia (d) and carcinoma (e) are quite similar to those arising in HER-2/*neu* transgenic mice. In d, the normal epithelium of a small lobular duct and the contiguous lobular structures are almost completely replaced by a solid occlusive proliferation of a relatively uniform population of round cells with a pale cytoplasm. (Hematoxylin and eosin staining; original magnification of a, c, d, e, f ×200; b ×100.)

Ultrastructural Examination

Ultrastructural examination of BALB-NeuT and FVB-NeuN tumors showed that most cells had a pale-staining, organelle-poor cytoplasm and a large oval nucleus with evenly distributed chromatin (Fig. 2, a, b, and d). Occasionally in BALB-NeuT and frequently in FVB-NeuN mice, tumor cells had darker cytoplasms and more irregular nuclei. The cells were linked by poorly developed junctions. In lobular carcinoma "in situ," a thin and discontinuous layer of myoepithelial cells and a basal lamina surrounded almost all tumor-containing ductules and alveoli. Invasive lesions were accompanied by a loss of myoepithelial cells and basal lamina. The histologic and ultrastructural features of these carcinomas were identical to those of human lobular carcinoma (Fig. 1, d and e, and Fig. 2c).

Immunohistochemistry

Immunohistochemistry with anti-*neu* antibody showed that the epithelial cells of extralobular ducts were mainly negative, whereas those of nonneoplastic lobular ducts and lobules and of neoplastic lobular lesions displayed a strong cell membrane staining (Fig. 3, a and b).

Proliferating cell nuclear antigen (PCNA) was expressed by the majority (65.2% ± 13.1%) of epithelial cells in hyperplastic ductular and lobular structures (Table 1 and Fig. 3c), whereas only 17.8% ± 3.1% of cells of extralobular ducts were positive. Its expression in lobular carcinomas (Fig. 3d) was mainly detected in the peripheral cell layer of neoplastic lobules (24.8% ± 7.3% of epithelial cells).

Intercellular E-cadherin expression was found in normal and hyperplastic mammary glands from both BALB and FVB transgenic mice. It was still detectable in BALB-NeuT, but not in FVB-NeuN lobular carcinomas (Fig. 3, e and f).

Studies were also performed in BALB transgenic mice to investigate angiogenesis during tumorigenesis. Before (2nd week) and during hyperplasia (5th week), and when lobular carcinoma in situ (15th week)

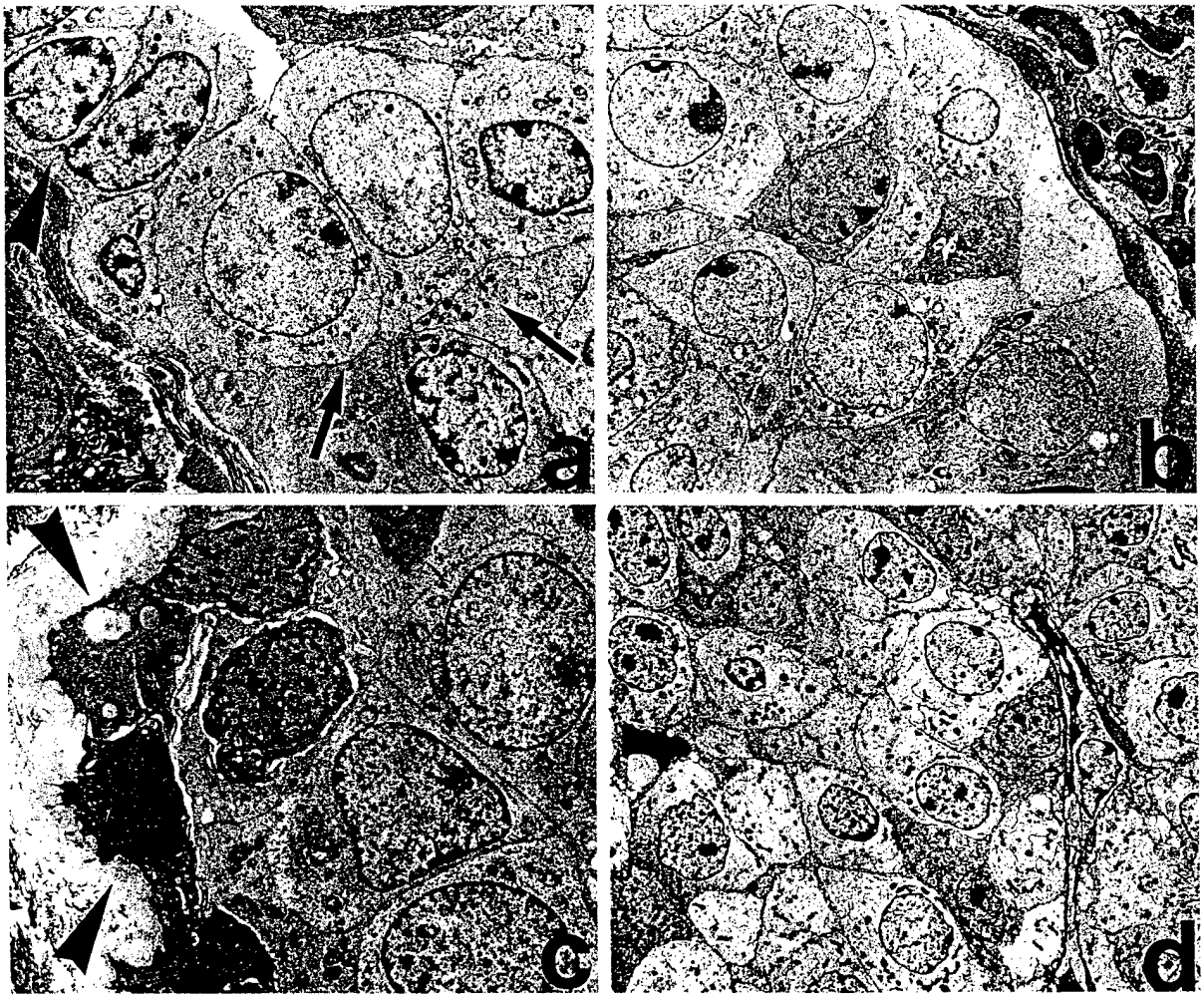

Figure 2.
Ultrastructural features of hyperplastic ductular structure in BALB-NeuT mice in which proliferating neoplastic cells (*arrows*) replace the normal epithelium (*arrowhead*) (a). Lobular carcinoma in BALB-NeuT (b) and FVB-NeuN mice (d) constituted of round to polygonal cells with an organelle-poor cytoplasm and a large round or oval nucleus (b). Human lobular carcinoma in situ (c) with neoplastic cells quite similar to those of mouse lobular carcinoma. Myoepithelial cells (*arrowheads*) lining the neoplastic lobular structure are tightly close to the basal lamina. (a, c original magnification, ×2,750; b ×1,900; d ×1,450.)

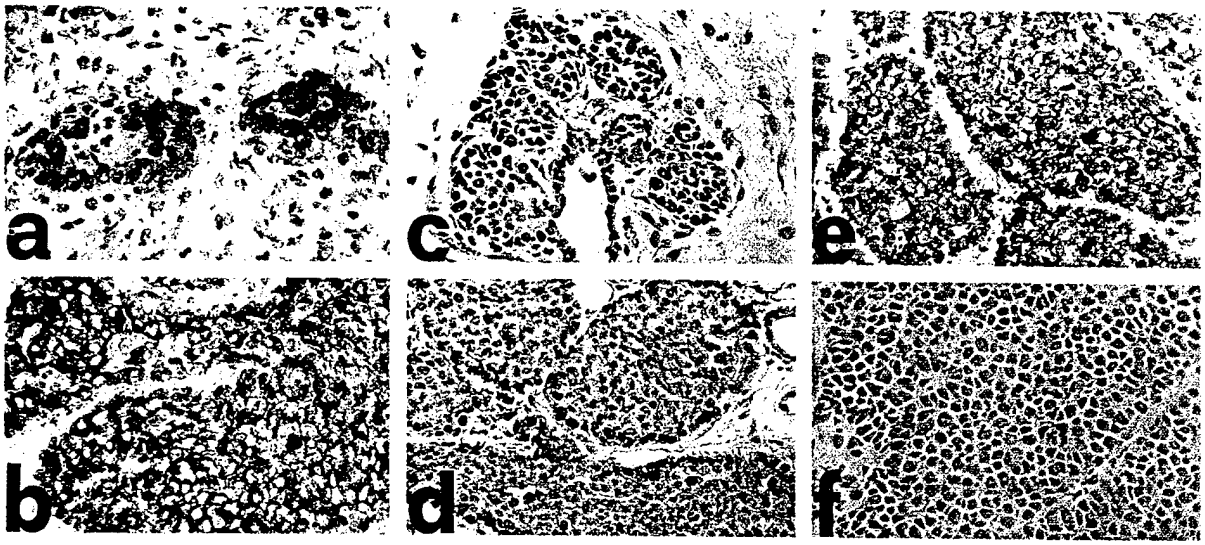

Figure 3.

Immunohistochemistry performed with anti-*neu* antibody revealed a strong positivity of epithelial cells in hyperplastic (a) and neoplastic (b) lobular lesions. Proliferating cell nuclear antigen is expressed by the majority of epithelial cells in hyperplastic ductular and lobular structures (c), whereas in lobular carcinoma it is mainly detected in the peripheral cell layer of neoplastic lobules (d). Intercellular E-cadherin expression is evident in lobular carcinoma of BALB-NeuT (e), but not in that of FVB-NeuN (f). (a–f original magnification, ×630.)

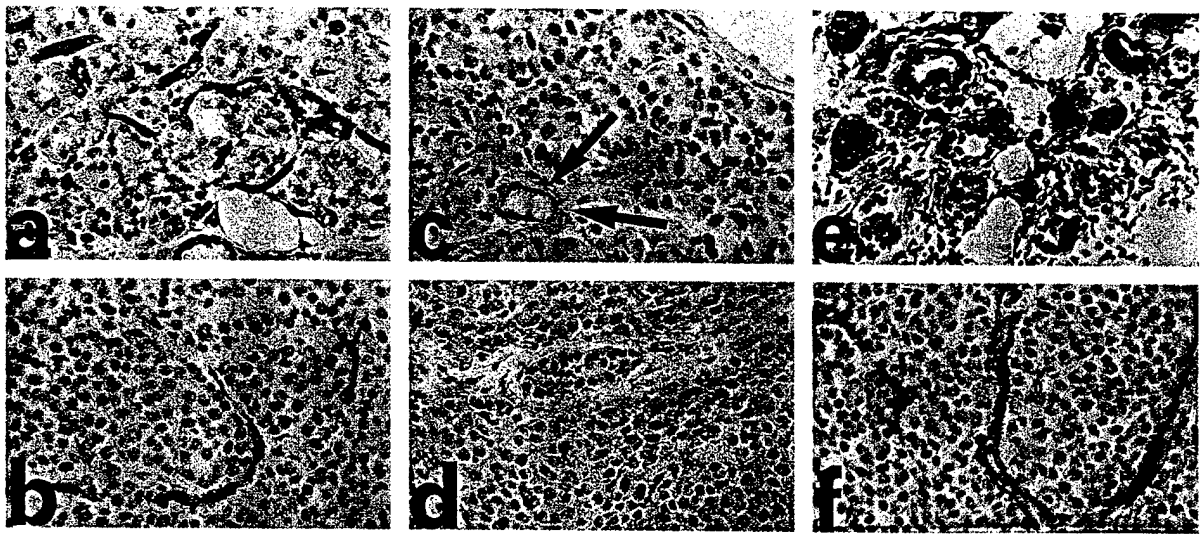

Figure 4.

Cryostat sections tested with anti-endothelial cells antibody (anti-CD31) showing that hyperplastic foci (a) are much more vascularized than carcinomatous mammary tissue (b). Some capillaries (*arrows*) present in hyperplasia (c) express the β_3 subunit of the $\alpha_v\beta_3$ receptor, which is almost absent in lobular carcinoma (d). Basic fibroblast growth factor (bFGF) is clearly expressed by epithelial cells during hyperplasia (e), whereas in lobular carcinoma, a marked bFGF staining is evident in the extracellular matrix bordering the neoplastic lobular structure (f). (a–f original magnification, ×630.)

and invasive alveolar lobular carcinoma (28th week) developed, tissue specimens were tested with anti-endothelial cells (CD31), anti-basement membrane components (anti-laminin and anti-collagen type IV), and anti-β_3 chain antibody, which recognizes the adhesion receptor $\alpha_v\beta_3$ selectively expressed on growing vessels.

Microvessel counts indicated that hyperplastic foci were much more vascularized than nonhyperplastic or carcinomatous tissue (Table 1 and Fig. 4, a and b). Several capillary sprouts in hyperplastic foci expressed the β_3 chain of the $\alpha_v\beta_3$ receptor (Fig. 4c) which was absent in normal mammary tissue. The capillary basement membrane component laminin showed a fibrillar distribution (data not shown) instead of the linear pattern found in quiescent mature vessels. A scanty presence of β_3 and a well-defined and continuous pattern of basal lamina components were observed in lobular carcinomas, in which both extracellular matrix molecules laminin and collagen type IV were more represented than in hyperplasia.

Immunohistochemical staining for angiogenic factors demonstrated that vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor (bFGF), absent or scarcely present in normal mammary tissue, were clearly expressed by epithelial

Table 1. Microvessel Count, Rate of Proliferating Cells, and Expression of β_3 Integrin, Angiogenic Factors, and Extracellular Matrix Components in Hyperplastic and Carcinomatous Tissues of BALB-NeuT Mice

	BALB-NeuT mice	
	Hyperplasia (5 wk of age)	Lobular carcinoma (15 wk of age)
Microvessel count	27.0 ± 3.2[a]	15.9 ± 2.1*
PCNA immunoreactivity rate	65.2% ± 13.1%	24.8% ± 7.3%*
β_3 Integrin	++[b]	+/−
bFGF	++	+
VEGF	+	+/−
Laminin	+	++
Collagen type IV	+	++

[a] Microvessel counts were performed on cryostat sections tested with anti-endothelial (CD31) Ab and the rate of proliferating cells (PCNA immunoreactivity) was evaluated on formalin-fixed, paraffin-embedded tissue sections with anti-PCNA Ab as described in Materials and Methods. At least 10 fields were counted per sample. Values are expressed as mean ± so of five 5-wk-old and five 15-wk-old mice.

[b] The expression of β_3 integrin, angiogenic factors, and extracellular matrix components was defined as absent (−), or scarcely (+/−), moderately (+), or frequently (++) present on cryostat sections tested with the corresponding antibody.

* Value significantly different ($p > 0.001$) than that of hyperplasia.

PCNA, proliferating cell nuclear antigen; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial cell growth factor.

cells during hyperplasia (Fig. 4e and Table 1). It also showed that their corresponding proteins were mainly confined in the basal neoplastic epithelial cell layer. A marked bFGF staining was evident in laminin and collagen type IV rich extracellular matrix bordering the neoplastic lobular structures (Fig. 4f).

Detection of Estrogen and Progesterone Receptors

A low level of estrogen receptors (ER) (8.1 fmole; range, 7.4 to 9.3) and progesterone receptors (PR) (16.2 fmole/mg cytosol protein; range, 14.8 to 17.7) was found in BALB-NeuT carcinomatous tissue. The K_d values for both receptors ranged from 0.1 to 0.2 nM.

Reverse Transcriptase-Polymerase Chain Reaction Analysis

Expression of bFGF and VEGF was also demonstrated by reverse transcriptase-polymerase chain reaction in BALB-NeuT carcinomatous tissue at the mRNA level (Fig 5).

Discussion

Expression of the activated rat HER-2/*neu* oncogene in BALB-NeuT mice results in the rapid and synchronous development of multifocal mammary tumors, whereas FVB-NeuN mice carrying the wild-type HER-2/*neu* oncogene develop mammary carcinomas asynchronously with a longer latency and lower multiplicity (Boggio et al, 1998; Muller et al, 1988). Despite these kinetic differences, histologic and ultrastructural ex-

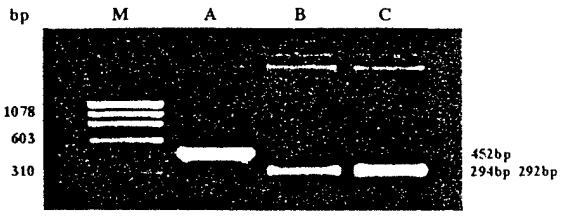

Figure 5.
Expression of vascular endothelial cell growth factor (VEGF) (294 bp; lane B) bFGF (292 bp; lane C) in mRNA extracted from BALB-NeuT lobular carcinoma. Total RNA was isolated from mammary tissue obtained from a 25-week-old mouse. The 292-bp bFGF band is wider than that of VEGF (294 b). G3DPH message (452 base pairs [bp]; lane A) served as control. The marker (lane M) is *Hae*III digest of Phi ×174.

amination of neoplasias indicates that expression of activated or overexpression of the wild type of rat HER-2/*neu* oncogene leads to the development of lobular carcinomas. This identity is also in agreement with the finding that the HER-2/*neu* proto-oncogene is frequently activationally mutated in FVB-NeuN transgenic mice (Siegel et al, 1994).

Diagnosis of these lobular carcinomas is based on replacement of the normal epithelium of acini and intralobular ductules by neoplastic cells. This conclusion is derived from our weekly histologic and immunohistochemical evaluation of HER-2/*neu* mammary tissue, which initially displays hyperplasia spreading all over the lobular structures, followed by an "in situ" and then an invasive lobular carcinoma. Furthermore epithelial proliferation within the lobular structures is characterized by solid, occlusive proliferation of a relatively uniform population of loosely cohesive, and mainly small, round cells with sparse cytoplasm distending the acini. At the ultrastructural level, the neoplastic cells display an organelle poor cytoplasm with oval, pale nuclei and inconspicuous nucleoli. These histologic and ultrastructural findings are identical to those observed in human mammary lobular carcinoma (Eusebi et al, 1977; Murad, 1971; Nesland et al, 1995; Rosen and Oberman, 1993). The acinar outlines remain distinct and separate from one another with persistence of intervening delicate stroma. It is important to note that the carcinoma in both strains is multicentric, as often reported in human lobular carcinoma.

Pathologists probably have not defined these tumors as lobular (Bouchard et al, 1989; Guy et al, 1996; Munn et al, 1995) because they mainly focused on the cytologic aspects of transformed epithelial cells which were identified as "intermediate cells" (Cardiff et al, 1991), ie, clear or basal cells supposed to originate from a metaplastic alteration occurring in mammary epithelial or myoepithelial cells. Furthermore the tendency of tumor cells to grow in a solid, loosely cohesive manner might be attributed to a relative, ultrastructurally observed, preservation of cell-to-cell junctions, that probably prevent the establishment of the "Indian file" arrangement and the "targetoid" growth pattern frequently found in invasive human lobular carcinoma (Rosen and Oberman, 1993). Cer-

tainly, the absence of these morphologic aspects does not make the diagnosis of lobular carcinoma easier. Moreover the occasional presence of central necrosis inside the neoplastic lobules mimics a characteristic feature of ductal mammary carcinoma (Rosai, 1996). A further reason for the lack of definite histologic characterization could be the frequent association in humans of HER-2/*neu* overexpression with ductal carcinoma (25% to 40%), whereas in lobular carcinoma this association is rarely found (1% to 13%) (Porter et al, 1991; Querzoli et al, 1998; Zschiesche et al, 1997).

In an attempt to better define the phenotypic profile of the transformed epithelial cells in mammary carcinoma of BALB-NeuT mice, we found a low level expression of ER and PR. Early studies in humans suggested that invasive lobular carcinoma was exceptionally ER-rich, but this has not been substantiated in larger groups (Lesser et al, 1981). High levels of ER and PR were found in 12 patients with the alveolar variant of invasive lobular carcinoma, though values ranged from more than 300 to 1495 fmole/mg of cytosol protein (Du Toit et al, 1989; Shousha et al, 1986).

The E-cadherin molecule is expressed at the surface of epithelial cells and plays a crucial role in epithelial organization and adhesion (Takeichi, 1991). Its expression frequently is reduced in human mammary lobular carcinomas (Vos et al, 1997), mainly in those with a more pronounced metastatic potential (Berx et al, 1995; Siitonen et al, 1996). Lobular carcinoma from BALB-NeuT mice strongly expressed E-cadherin, whereas it was almost undetectable in FVB-NeuN mammary tumors. This latter feature could explain why Guy et al (1992) found that the overexpressed rather than the activated HER-2/*neu* gene enhances the metastatic potential of the mammary tumor cell.

The major functional units of the mouse mammary gland are termed lobulo-alveolar units or terminal end buds, which are regarded as equivalent to the terminal ductal lobular units of the female human breast (Cardiff, 1998; Russo and Russo, 1996).

There is evidence that spontaneous and chemically induced ductal tumors develop in lobulo-alveolar/terminal end buds units (Cardiff, 1998; Russo and Russo, 1996). Because these units contain the proliferative stem cell populations most sensitive to the effects of somatic cell mutation, they seem to be the site of origin for most mammary cancers, including those of lobular type. It has been hypothesized that human lobular carcinoma arises from a more complex and differentiated lobular structure (lobule type 2) that evolves from terminal ductal lobular units (Russo and Russo, 1996).

In BALB and FVB transgenic mice, the genetic alteration may lead to proliferation of epithelial cells in the already well-differentiated lobular structures, similar to lobule type 2, which contains almost all cells expressing HER-2/*neu* product. HER-2/*neu*–triggered epithelial cell proliferation is evidenced by the widely distributed expression of PCNA in these more differentiated lobular structures. Conversely, neoplastic cell proliferation starting in the lobulo-alveolar/terminal end buds units may give rise to the more complex lobular arrangement. This pathogenic pathway is probably based on the multifocal and widely distributed presence of HER-2/*neu*–expressing transformed cells.

Previous studies in transgenic mice reported that tumorigenesis proceeds through two stages (Folkman et al, 1989; Parangi et al, 1996). The first involves oncogene product expression which leads to hyperplasia, the second consists of angiogenesis induction. Our findings provide a further illustration of this pattern. In our model there seems to be a close connection between hyperplasia, characterized by an increase in epithelial cell proliferation, and the activation of angiogenesis. We have morphologic evidence that in hyperplastic foci, angiogenesis begins before overt tumor formation. In hyperplasia, we observed an increased number of microvessels in the stroma surrounding the hyperplastic small lobular ducts and lobules. Several microvessels expressed the b_3 subunit of $\alpha_v\beta_3$ integrin, which has been reported to promote endothelial cell migration, angiogenesis, and protection from apoptosis (Brooks et al, 1994a, 1994b; Shattil, 1995). Its expression identifies new vessel sprouts and is a real indicator of neovascularization (Brooks et al, 1994a).

Neovascularization is probably activated by bFGF- and VEGF-producing hyperplastic epithelial cells. These angiogenic factors were also expressed in lobular carcinoma confined to the basal neoplastic epithelial cell layer close to the intervening stroma. It has been reported that bFGF molecules stored and immobilized in the extracellular matrix are normally inactive because of their strong adherence to heparin sulfate proteoglycans (Czubayko et al, 1997; Rak and Kerbel, 1997). During tumor progression, therefore, the extracellular matrix could sequester bFGF and impede its angiogenic effects. The mean number of microvessels per microscopic field, in fact, was appreciably reduced in lobular carcinoma compared with the preceding hyperplasia, in which the extracellular matrix constituents (laminin and collagen type IV) were less represented.

Discussions on spontaneous or chemically induced mammary tumors have never reported a lobular type of carcinoma in rodents (Munn et al, 1995; Russo and Russo, 1996), whereas the ductal type has been widely and perhaps solely described.

Apart from two casual observations (Kordon et al, 1993; Pazos et al, 1998), the finding of a lobular carcinoma in the two strains of transgenic mice studied in this work, adds a new histotype to the current histologic classification of rodent mammary epithelial neoplasms. Diagnosis in rodents of a lobular carcinoma of the alveolar type resembling that occurring in women, and our finding of its peculiar pattern of neoangiogenesis may be considered a substantial clue for anticancer research and supply an appropriate tool for the testing of new therapeutic strategies.

Materials and Methods

Mice

A transgenic CD1 random-bred breeder male mouse (no. 1330) carrying the mutated rat HER-2/*neu* oncogene driven by the mouse mammary tumor virus promoter (Tg-NeuT, provided by Dr. L. Clerici, Euratom, Ispra, Italy) (Lucchini et al, 1992) was mated with BALB/c females (H-2^d; Charles River, Calco, Italy). The progeny was screened for the transgene by PCR. Transgene-carrying males were backcrossed with BALB/c females for more than 12 generations and HER-2/*neu* BALB/c mice (BALB-NeuT) were used in these experiments. Parental FVB-NeuN N#202 transgenic mice carrying the rat HER-2/*neu* proto-oncogene driven by the mouse mammary tumor virus promoter on the H-2^q FVB inbred background were provided by Dr. W. J. Muller (McMaster University, Hamilton, Ontario, Canada) and bred in our animal facilities. Females of both lines show a mouse mammary tumor virus-driven overexpression of the transgene in the mammary gland and a definite tumor growth involving its epithelium (Guy et al, 1992, 1996; Lucchini et al, 1992). Individually tagged virgin females were used in this study. Starting at the age of 5 weeks, their mammary glands were inspected once a week, and masses were measured with calipers in the two perpendicular diameters (Guy et al, 1992). Progressively growing masses >3-mm mean diameter were regarded as tumors.

Histologic and Ultrastructural Analysis

Groups of two or three BALB-NeuT mice were killed at Week 2 and 3 and then every other week until Week 33 of age; similar groups of FVB-NeuN were killed every 4 weeks from 5 to 61 weeks of age. For histologic evaluation, tissue samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin or Giemsa. For electron microscopy, specimens were fixed in cacodylate-buffered 2.5% glutaraldehyde, postfixed in osmium tetroxide, and then embedded in Epon 812. Ultra-thin sections were stained with uranyl acetate-lead citrate.

Immunohistochemistry

For immunohistochemistry, formalin-fixed, paraffin-embedded or acetone-fixed cryostat sections were incubated for 30 minutes with anti-endothelial cells (mEC-13.324) (Vecchi et al, 1994), anti-CD61 (integrin β_3 chain) (PharMingen, San Diego, California), anti-collagen type IV (Chemicon, Temecula, California), anti-laminin (Becton Dickinson, Bedford, Massachusetts), anti-VEGF, anti-bFGF and anti-Neu (C-18) (Santa Cruz Biotechnology, Inc., Santa Cruz, California), anti-PCNA (Ylem, Rome, Italy), and anti-uvomorulin (E-cadherin) (Sigma Immunochemicals, Milan, Italy) antibodies. After washing, the cryostat sections were overlaid with biotinylated goat anti-rat, anti-hamster, and anti-rabbit or horse anti-goat Ig (Vector Labs., Burlingame, California) for 30 minutes. Unbound Ig was removed by washing and the slides were incubated with avidin-biotin complex/alkaline phosphatase (Dako, Glostrup, Denmark). Quantitative studies of immunohistochemically stained sections were performed independently by three pathologists in a blinded manner. Two or more samples (1/tumor growth area) and 10 randomly chosen fields in each sample from mice with multiple hyperplastic foci or tumors were evaluated for each point determination. For microvessel and reactive cell counts, individual microvessels and cells were counted under a microscope ×400 field (×40 objective and ×10 ocular lens; 0.180 mm^2 per field). The rate of immunoreactivity for PCNA was obtained by counting the number of positive cells/number of total cells in the ductular and lobular structures under a microscope ×600 field (×60 objective and ×10 ocular lens; 0.120 mm^2 per field).

The expression of β_3 integrin, angiogenic factors, and extracellular matrix components was defined as absent (−), scarcely (+/−), moderately (+), or frequently (++) present on cryostat sections tested with the corresponding antibodies.

Estrogen and Progesterone Receptors

ER and PR were assessed as reported by Carbone and Vecchio (1985) using the dextran-coated charcoal method, as recommended by the Italian Committee for Standardisation of Tissue Hormonal receptors assays (Piffanelli et al, 1982). The concentration and apparent equilibrium dissociation constant (K_d) of receptor sites were obtained by Scatchard analysis.

mRNA for Angiogenic Factors

Total RNA was prepared from BALB/c normal mammary tissue and from BALB-NeuT neoplastic lesions by using Ultraspec (Biotecx Laboratories, Inc., Houston, Texas). Two micrograms of RNA were reverse transcribed with Moloney murine leukemia virus reverse transcriptase (200 U) in 50 μl of reaction mixture with oligo dT and dNTP (GIBCO BRL, Paisley, United Kingdom). The cDNA were tested for the presence of murine glucose 3-phosphate dehydrogenase, VEGF, and bFGF sequences in PCR reactions (Gene Amp Kit; Perkin Elmer Cetus, Norwalk, Connecticut) performed in 20-μl volumes and amplified by 30 PCR cycles, by using specific primer pairs prepared by us (VEGF) or from Stratagene (La Jolla, California) (bFGF).

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Specific and nonspecific immunity in the prevention of spontaneous tumours

Pier-Luigi Lollini and Guido Forni

Antitumour vaccines cannot cure established tumours. However, tumour cells engineered to release cytokines can halt progression to cancer by inhibiting the angiogenic phenotype of pretumoural cells and activating tumour-associated leukocytes, thus they might prove useful in immunizing those at risk of cancer.

The presentation of tumour-associated antigens by dendritic cells demonstrates that defined peptides can elicit a specific antitumour immunity[1]. The recognition of multiple dominant and subdominant tumour antigens by the immune system can be evoked by whole tumour cells engineered with adhesion and costimulatory molecules, suicide genes or cytokines (reviewed in Ref. 2). When a tumour is engineered to release cytokines, it is the type of cytokine released that decides which immune mechanisms are elicited in a privileged way. Thus, selective activation of cytokines that are most appropriate to the stages of tumour progression is feasible[3].

These vaccine strategies are often effective. Tumours that in more conventional ways are unable to induce significant immune responses, evoke them when their antigens are presented by dendritic cells[1,2] or their engineered cells are used for vaccination[2,3]. This has altered our approach to the immunoregulation of tumours. However, the usual pitfall in evaluating the potential of these new vaccines is to overlook the fact that abrogation of the tumourigenicity of gene-engineered cells, and effective immunization of healthy animals against subsequent challenge by wild-type tumour cells, have little to do with therapy[4]. Their real ability to cure existing tumours has hardly ever been investigated. In most studies, only a minority of tumour-bearing mice were cured, and this limited efficacy was solely achieved when the vaccine was administered in the first few days after challenge[5]; corresponding clinical trials have not disclosed any significant ability to cure[6].

Nonetheless, further consideration must be given to the inability of these vaccines to prevent tumours. It may well be that their failure is too bitter a notion to swallow and hence is either consciously or unconsciously ignored. Several objectives have been made possible by these vaccines (Box 1), and the cure of clinically evident tumours has been the most implausible and, at the same time, the most common goal in clinical trials. The many ethical and emotional issues raised by cancer provide the main reason for these clinical attempts. Paradoxically, their failure is often blamed on the poor reliability of results from mouse models. In addition, more efficacious vaccines are called for. Experimental models suggest that vaccines engineered with combinations of genes are more effective than those with one only. However, to date, there is no clinical evidence to support this view[6].

Here, we emphasize that the high degree of immunogenicity that these new vaccines can offer is 'far too weighty a baby to be simply thrown out with the bathwater'. Improved vaccination is perhaps less important than the realization that the mechanisms elicited by specific immunization are not suited to cure established tumours. This issue is underscored, first, by the demonstration that these mechanisms lead to the rejection of normal tissues, but not to the rejection of tumours expressing the same target antigen[7]; and second, by the many ways

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Box 1. What can be gained by interfering with the tumour–host immune relationship?

- Induction (or increase) of surveillance against tumour onset
- Induction of resistance to minimal residual disease and tumour recurrences
- Restraining the progression of clinically evident tumours
- Successful treatment of clinically diagnosed tumours

in which an established tumour manages to elude these immune mechanisms [F. Cavallo, (1997) *Immunological Blackboard* (Vol. 1, No. 1) http://pages.inrete.it/immunoblack]

Vaccination is a distinct example of preventive medicine, whereas 'therapeutic vaccination' is a distorted concept that has had no great success, even in the handling of infectious diseases. Despite this, the experimental data indicate that cancer vaccines should be able to cope with minimal residual disease, prevent recurrences and inhibit incipient metastases after conventional tumour management[8].

Tumour prevention by specific immunity

Recent studies have led to the discovery of gene mutations that predispose to cancer. Thus, it might be possible to detect susceptible individuals with a defined genetic prognosis[9]. Identification of the gene at risk and its mutated or amplified products would provide a heaven-sent opportunity to vaccinate susceptible subjects against their foreseeable cancer. The molecular characterization of altered gene products predicted to become tumour antigens will be the first step towards engineering effective vaccines for this purpose.

Identification of human tumour antigens has revealed that a few of them are expressed by distinct tumours[10]. If the most common antigens are found to number ~50, vaccination of healthy individuals against tumours will become more feasible. Immunologic intervention has been clearly shown to prevent the onset of virus-related tumours, such as Marek's disease of poultry[11], and human hepatocellular carcinoma[12], where vaccination prevents cancer by eliminating the main risk factor.

The question whether immunologic approaches can be successful once a cell population has been subjected to the initial carcinogenic hit, has rarely been examined. However, vaccination could plausibly induce a strong immune response against ignored or fully tolerated antigens associated with the most common tumours that arise within a population. Owing to the polymorphism of the glycoproteins of the major histocompatibility complex (MHC), different vaccines would need to be prepared to fit the polymorphic peptide-binding clefts. It is likely that certain tumour antigens will have a restricted usage and a few individuals will not be easily vaccinated.

These are practical and perhaps solvable problems. The real issue is whether inducing an efficient immune response will offer protection against spontaneous tumours. The central tenet of tumour immunology is that recognition of tumour antigens is followed by the establishment of a long-lasting immune memory and the specific killing of tumour cells. This notion is supported by experimental data from many transplantable tumours. The use of appropriate vaccines has shown that even spontaneous tumours, originally thought to be nonimmunogenic[13], can induce protection against subsequent challenge[14]. Nevertheless, very few data are available on spontaneous tumours, which display a longer and more complex natural relationship with their host than transplantable forms.

Mice transgenic for oncogenes might act as models to explore the defensive role of the immune system in tumourigenesis. For example, mice transgenic for the rat *neu* oncogene are protected from tumour development when vaccinated with the DNA encoding the extracellular domain of the *neu* p185 product[15] and soluble p185 protein[16], suggesting that they hamper the onset of tumours. The challenge is to pass from a proof of principle to an effective human vaccine.

Antigen-loss variants are unlikely to emerge as tumour escape mechanisms when the target molecule is directly linked to neoplastic transformation and progression, as in the case of p185 (P-L. Lollini *et al.*, unpublished) and other oncogene products. A more probable escape route is offered by the defects in antigen processing and MHC class I downregulation detected in murine[17] and human carcinomas[18].

Nonspecific immunity strikes back

Blockade of tumour growth through nonspecific stimulation of the immune system is a notion as old as it is naive. The molecular definition of many nonspecific reaction mechanisms, however, has corrected many prejudices. Straightforward comparison shows that nonspecific mechanisms possess a much greater curative potential than those elicited by specific immunity. Only a minority of mice challenged with an aggressive mammary carcinoma (TSA) were cured by repeated immunizations with cytokine-gene-engineered TSA cells[3,5] or TSA peptide-pulsed dendritic cells[1] begun immediately after the challenge, and almost none were cured when immunizations began on day 7 – however, the great majority of these 7-day-old tumours were cured by repeated injection of low doses of interleukin 12 (IL-12)[5,8]. Tumour destruction results from three major mechanisms: (1) destruction of tumour vessels by polymorphonuclear leukocytes; (2) indirect inhibition of angiogenesis by secondary interferon γ (IFN-γ), tumour necrosis factor α (TNF-α) and third-level chemokines; and (3) activation of leukocyte subsets capable of producing proinflammatory cytokines, cytotoxic T lymphocytes and antitumour antibodies[8].

Surprisingly, similar IL-12-triggered mechanisms inhibit both chemical[19] and *neu*-dependent[20] carcinogenesis. When BALB/c mice were injected subcutaneously with 3-methylcholanthrene, 100 ng IL-12 administered systemically 5 days/week for 18 weeks (3 weeks on, 1 week off) delayed tumour appearance and reduced tumour incidence. Secondary IFN-γ, IL-10 and TNF-α were induced throughout the treatment. High production of IFN-γ by $CD8^+$ T cells, and a T helper 2 (Th2) to Th1 or Th0 shift in the cytokine secretion profile of $CD4^+$ T cells were also seen in the treated mice[19].

The mammary glands of female BALB/c mice carrying the activated HER-2/*neu* oncogene and adult female FVB mice carrying the HER-2/*neu* protooncogene progress through atypical hyperplasia to *in situ* and

invasive lobular carcinoma. This progression begins in BALB/c-*neu* mice when they are weaning and in FVB-*neu* mice when they are adults. Systemic treatment of mice with preneoplastic lesions with IL-12 5 days/week (1 week on, 3 weeks off; first course 50 ng IL-12/day, the remainder 100 ng/day) markedly delayed tumour onset and reduced tumour multiplicity. Analogous results were obtained in immunocompetent and permanently $CD8^+$ T-cell-depleted mice. In both transgenic lines, tumour inhibition was associated with mammary infiltration by reactive cells, production of cytokines and inducible nitric oxide synthase (iNOS), reduction in microvessel number and a high degree of haemorrhagic necrosis[20].

These experiments suggest that stimulation of nonspecific immunity can prevent tumour formation – an unexpected and indeed provocative deduction. The resemblance of methylcholanthrene and HER-2/*neu* carcinogenesis models to human tumours indicates that nontoxic IL-12 regimens might constitute a significant prophylactic strategy. Generalization of these findings to other tumours and cytokines could allow stimulation of nonspecific immunity to be used to protect individuals with a genetic risk of cancer (Fig. 1a) and those with preneoplastic lesions (Fig. 1b,c) as a 'soft' immunologic alternative to controversial and distasteful preventive measures[9].

Apparently, IL-12 inhibits cancerogenesis by slowing down the transition from preneoplastic to overt tumours, halting angiogenesis and activating tumour-associated leukocytes through the induction of several secondary cytokines and mediators[19,20]. Nonspecific immunity can probably never lead to tumour eradication, particularly because some of its effector mechanisms, including its anti-angiogenic effects, are cytostatic rather than cytotoxic. They certainly appear to delay the appearance of tumours and, in some human situations, this could almost be regarded as equivalent to a cure[21]. Many chemopreventive agents are currently under investigation, including several new selective oestrogen receptor modulators[22]. In the future, combined chemical and immunologic preventive management might significantly decrease the incidence of clinically evident tumours in individuals at risk.

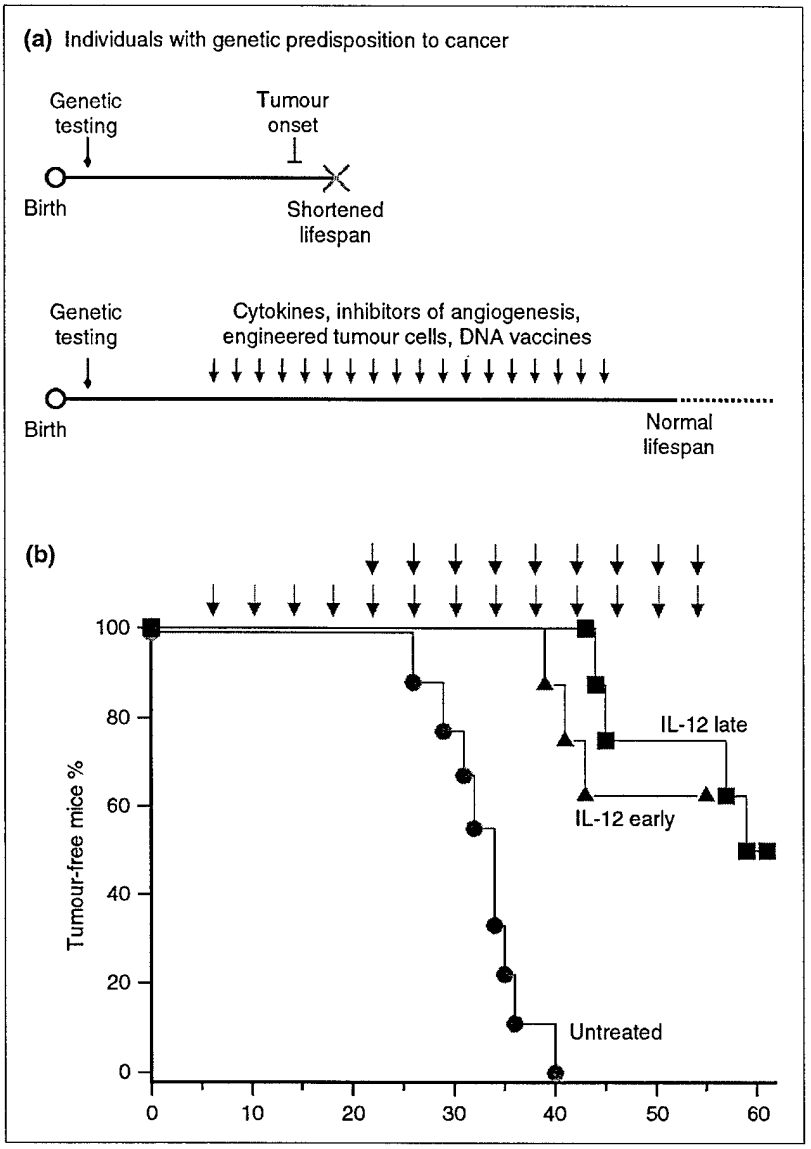

Fig. 1. *(a) The tumour prevention modalities currently available to individuals inheriting a genetic predisposition to cancer are distasteful and controversial[9,23]. An alternative is to interrupt tumour progression by manoeuvres that stimulate nonspecific host immune responses and inhibit tumour angiogenesis[19,20]. The graph (b) depicts an experimental proof of this concept. Transgenic FVB mice expressing the HER-2/neu protooncogene in the mammary gland invariably develop malignant carcinomas with a long latency period. A chronic treatment with recombinant interleukin 12 (rIL-12; each arrow represents one week of IL-12 treatment) significantly reduced tumour incidence (for further details, see text and Ref. 20). Tumour progression was prevented by IL-12 treatment started both in young mice (IL-12 early) and in adult mice (IL-12 late). In this combination of cancer predisposition and immunoprophylactic approach, a lifetime treatment was not necessary, thus sparing potentially harmful side-effects during young age.*

Conclusions

Molecular data on tumour antigens and elucidation of the protective role of the immune system in tumourigenesis might provide new strategies in oncology. The immunotherapeutic path trodden so far has not had much experimental backing and may be too hard to follow; its therapeutic success has been undeniably marginal. Nevertheless, it was fully

justified by the seriousness of the problem and led to many scientific discoveries. Development of antitumour vaccines and investigation of the defensive role of nonspecific immunity in tumourigenesis will not be easy. However, it might be rewarded by the creation of effective tumour prevention strategies.

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[CANCER RESEARCH 60, 359–364, January 15, 2000]

Appendix #3

Ability of Systemic Interleukin-12 to Hamper Progressive Stages of Mammary Carcinogenesis in HER2/*neu* Transgenic Mice[1]

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ABSTRACT

Previous studies in mice have shown that chronic administration of recombinant interleukin-12 (IL-12) hampers the progression of both chemical- and oncogene-dependent carcinogenesis. This suggests that a new preventive strategy may be envisaged for individuals with a genetic risk of cancer or carrying preneoplastic lesions. Starting at progressive stages of mammary carcinogenesis, female BALB/c and FVB mice carrying the activated rat HER2/*neu* oncogene (BALB-neuT) or the proto-oncogene (FVB-neuN) under the mouse mammary tumor virus promoter received multiple 5-day courses of different doses of IL-12. The times of tumor appearance, multiplicity, and histopathological features of the neoplastic lesions were evaluated. In both BALB-neuT and FVB-neuN mice, 5-day i.p. courses of 50/100 ng of IL-12/day inhibited mammary carcinogenesis when they coincided with the progression of early preneoplastic lesions. Inhibition appears to depend primarily on the ability of IL-12 to interfere with early tumor angiogenesis. Later treatments are much less effective, and daily doses of 10 and 2 ng are useless. The efficacy of early IL-12 courses suggests that they could be used to prevent mammary tumors in individuals at risk, whereas their lower efficacy in later stages of carcinogenesis and the dose range required pose some constraints on their use in the management of overt preneoplastic lesions. Precise understanding of tumor progression means that effective treatments can be commenced relatively late in the life of individuals at risk and that no lifetime administration is required.

INTRODUCTION

The remarkable ability of systematically injected recombinant IL-12[3] to inhibit transplantable mouse tumors (1–6) appears to rest on its induction of IFN-γ (2, 4), tumor necrosis factor α (5), and granulocyte/macrophage colony-stimulating factor (6). These secondary cytokines then induce other downstream factors that trigger a complex antitumor reaction. By acting on the endothelial cells of newly formed vessels, these mediators inhibit tumor neoangiogenesis (7, 8), induce the expression of adhesion molecules, and recruit leukocytes at the tumor site (7, 9). They also favor the elicitation of cytolytic effector cells and antitumor antibodies (3, 7, 10–12), whereas their presence in the tumor microenvironment affects tumor cells directly by inducing the overexpression of MHC glycoproteins (13) and switching the production of angiogenic factors to that of antiangiogenic factors (14).

IL-12 also hampers the progression of both chemical-(15) and *neu* oncogene-dependent (16) carcinogenesis and would thus seem open to exploitation as a preventive agent (17) because genetic screening is singling out individuals with a defined genetic risk of cancer (18), and preneoplastic lesions are being detected by early diagnosis programs (19).

To determine the stage of mammary carcinogenesis in which IL-12 most successfully inhibits the progression of preneoplastic lesions into invasive tumors, we used females of two transgenic mouse strains expressing the rat HER2/*neu* oncogene in the mammary gland. Although temporally differentiated by their kinetics, these two models of progression through atypical hyperplasia to *in situ* carcinoma and invasive carcinomas closely reproduce a few features of mammary carcinogenesis in women (16).

MATERIALS AND METHODS

Mice. BALB/c mice overexpressing the activated rat HER2/*neu* oncogene driven by the mouse mammary tumor virus (MMTV) promoter (Ref. 20; BALB-neuT) in their mammary glands were bred in our animal facilities (for details, see Ref. 16). A colony of FVB mice (N#202) carrying the rat HER2/*neu* proto-oncogene driven by the MMTV promoter (Ref. 21; FVB-neuN) was maintained under strict inbreeding from breeding pairs obtained from Dr. W. J. Muller (McMaster University, Hamilton, Ontario, Canada) as described previously (16). Groups of individually tagged virgin females were used. Their mammary glands were inspected weekly, and tumor masses were measured with calipers in two perpendicular diameters (16). Progressively growing masses of >3 mm in mean diameter were regarded as tumors. Growth was monitored weekly until all 10 mammary glands displayed a palpable tumor or until one tumor exceeded an average diameter of 1.5 cm, at which time mice were sacrificed for humane reasons. Surviving BALB-neuT mice were sacrificed at the 33rd week, when tumor masses were evident in all 10 mammary glands; FVB-neuN mice were sacrificed at 90 weeks, when they displayed a mean number of 2.5 tumors/mouse.

IL-12 Administration. IL-12 (Genetics Institute, Cambridge, MA) in HBSS supplemented with 0.01% MSA (Sigma, St. Louis, MO) was administered i.p. At the times indicated, mice received seven 5-day courses of MSA only (MSA controls) or MSA plus IL-12. Other groups of mice remained untreated. Because no appreciable differences in tumor growth rate and pathological findings were found between the untreated mice and the MSA controls, only the data of the latter group are shown. The first course consisted of 50 ng of IL-12/day, and the subsequent six courses consisted of 100 ng of IL-12/day. These seven courses were administered at different times (Fig. 1). BALB-neuT mice assigned to the chronic treatment group received the first course at the 2nd week of age. From the 5th to the 25th week, courses were repeated every 4th week. Mice assigned to the late treatment group received the courses from the 13th to the 25th week. They were treated for 2 consecutive weeks, followed by 2 weeks off. Mice in the early treatment group received IL-12 beginning at the 2nd week and ending at week 14. In a few experiments, the early treatment was also performed with 10 and 2 ng in all seven courses. FVB-neuN mice received the courses every 4th week, starting on the 6th (6-week-old treatment), 22nd (22-week-old treatment), or 28th (28-week-old treatment) week of age. All of these treatments continued until week 90.

Histological and Immunohistochemical Analysis. Groups of three IL-12-treated and untreated BALB-neuT mice were killed at 15, 25, and 30 weeks of age, whereas similar groups of FVB-neuN mice were sacrificed at weeks 15, 20, 22, 25, 27, and 30. For histological evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm,

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[3] The abbreviations used are: IL, interleukin; MSA, mouse serum albumin; MMTV, mouse mammary tumor virus; PCNA, proliferating cell nuclear antigen.

and stained with H&E or Giemsa. For immunohistochemistry, formalin-fixed, paraffin-embedded, or acetone-fixed cryostat sections were incubated for 30 min with antiendothelial cells (mEC-13.324; Ref. 22) and PCNA (Ylem, Rome, Italy) antibodies. After washing, the cryostat sections were overlaid with biotinylated goat antirat and mouse antigoat IgG (Vector Laboratories, Burlingame, CA) for 30 min. Unbound antibodies were removed by washing, and the slides were incubated with avidin-biotin complex/alkaline phosphatase (DAKO, Glostrup, Denmark). Quantitative studies of immunohistochemically stained sections were performed independently by three pathologists in a blind fashion. Two or more samples (one per tumor growth area) and 10 randomly chosen fields in each sample from mice with multiple hyperplastic foci or tumors were evaluated for each determination. Individual microvessels were counted under a microscope ×400 field (×40 objective and ×10 ocular lens; 0.180 mm^2 per field). The rate of immunoreactivity for PCNA was obtained by counting the number of positive cells/number of total cells in the ductular and lobular structures under a microscope ×600 field (×60 objective and ×10 ocular lens; 0.120 mm^2 per field).

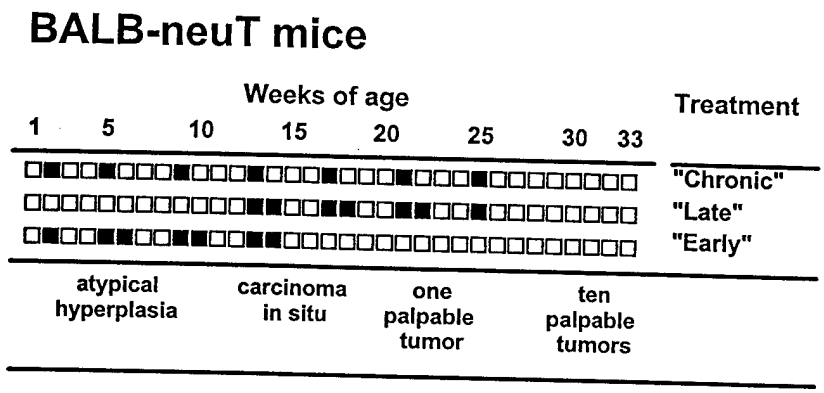
Statistical Analysis. Differences in tumor incidence were evaluated by the Mantel-Haenszel log-rank test; differences in tumor/mouse numbers, the number of microvessels, and PCNA immunoreactive cells were evaluated by Student's *t* test.

RESULTS

IL-12 Delay of Carcinogenesis in BALB-neuT Mice. With a slightly asynchronous but consistent pattern, all mammary glands of untreated and MSA control BALB-neuT female mice progress into invasive carcinoma (Fig. 1; Ref. 16). Atypical hyperplasia of small lobular ducts and lobules is already evident at the 2nd week of age. At the 10th week, proliferating epithelial cells occlude the ductules and acini within the lobules. Vigorous capillary proliferation is evident at the 15th week, when atypical hyperplasia is prominent, often assuming the aspect of carcinoma *in situ* (Fig. 2*a*). Near the 20th week, the neoplastic ductular-lobular structures progressively expand and invade the surrounding tissues, and at least one palpable tumor mass is detectable around the 19th week (Fig. 3, *bottom panel*). Invasive lobular carcinomas (Fig. 4*a*) develop progressively, and at the 33rd week, tumor masses are palpable in all 10 mammary glands.

To evaluate the ability of IL-12 to inhibit this progression, mice received seven 5-day courses of IL-12 at different times (Fig. 1). In the chronic treatment, the courses started in the 2nd week and continued until the 25th week. Both a delay in the onset of the first mammary tumor and a 50% reduction in the number of mammary glands with a palpable tumor at 33 weeks (when the experiment was ended) were observed as compared with MSA controls (Fig. 3). To assess whether IL-12 is also effective during later phases, other mice were first treated at the 13th week of age, when hyperplasia takes the form of a carcinoma *in situ*. Courses continued until the 25th week. This late treatment did not delay the onset of the first tumor but did reduced the number of tumors at week 33 by 22%. The early treatment began at the 2nd week and continued until week 14. The delay in onset of the first tumor and the reduction in the number of tumors are significantly higher than those seen in the chronic treatment group. When the early treatment was further split into shorter 4-week administration schedules, much less protection was observed (data not shown).

Pathology of Mammary Lesions in BALB-neuT Mice. A similarly widespread atypical hyperplasia of small lobular ducts and lobules with multiple foci of carcinoma *in situ* was evident at week 15 in the MSA controls and in the late treatment group that had received two IL-12 courses only at that time. However, in the latter group distinct vascular damage associated with few reactive cells close to hyperplastic and neoplastic lobules was evident. Mice from the chronic and early treatment groups revealed a less widely distributed



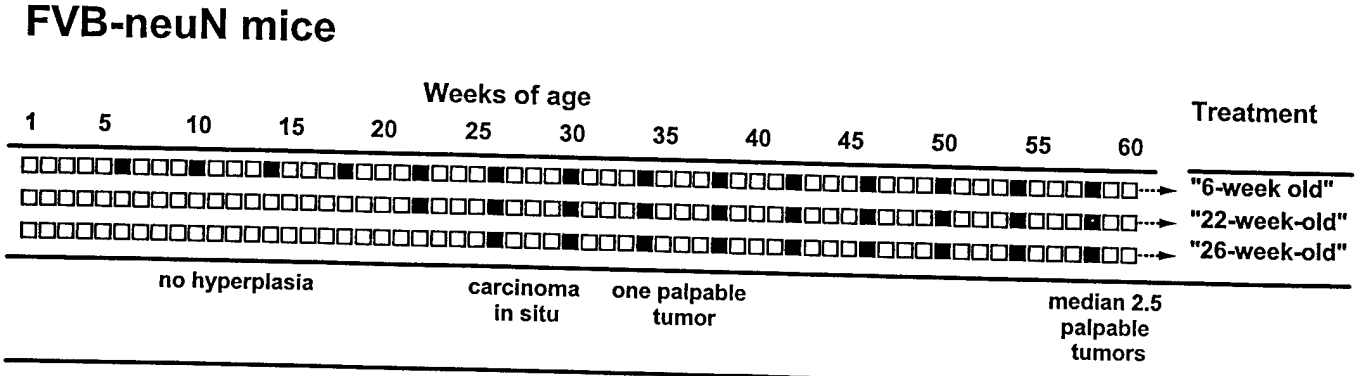


Fig. 1. Treatment outline. ■, weeks in which mice received 5-day courses (Monday through Friday) of daily i.p. injections of IL-12 or MSA only during the progression of HER2/*neu* mammary carcinogenesis.

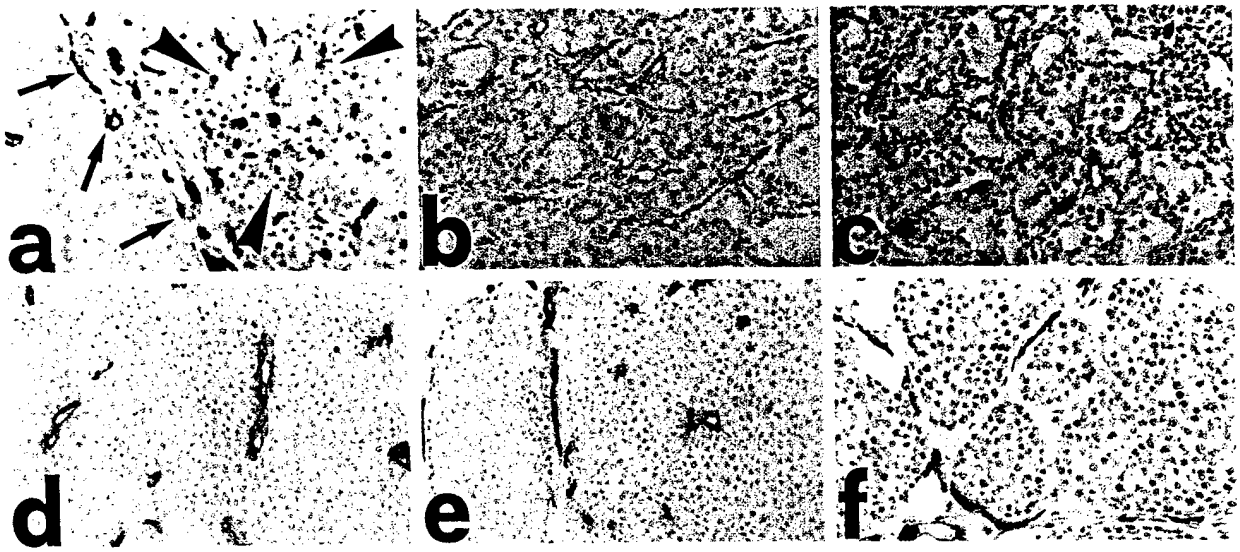


Fig. 2. Vascularization of mammary lesions in BALB-neuT mice. At 15 weeks, mammary glands from MSA control mice display numerous capillary sprouts (*arrowheads*) inside atypical hyperplastic areas, whereas scanty vascularization (*arrows*) is present at the periphery of *in situ* carcinoma (*a*). In hyperplastic mammary tissue from mice in the chronic treatment group, a clear reduction in the number of microvessels is evident (*b*). Mice from the early treatment group (*c*) display a marked reduction associated with a defective vascular network. At 25 weeks of age, the differences in the vascular architecture of the neoplastic lesions from the MSA control mice (*d*), chronic (*e*), and early (*f*) treatment groups are less evident.

atypical hyperplasia. Rare foci of carcinoma *in situ* were present in tissues from mice of the chronic treatment group, but not in those from the early treatment group (data not shown). At week 25, invasive carcinomas were present in the MSA controls (Fig. 4*a*). At this time, the IL-12 regimens resulted in distinct pathological features. Either *in situ* carcinomas or invasive carcinomas were evident in the mammary glands of mice from the chronic and late treatment groups (Fig. 4,*b* and *c*). These lesions were smaller and less widely distributed than those in MSA controls and were even less pronounced in the chronic treatment group. In contrast, a restrained atypical hyperplasia with foci of carcinoma *in situ* only was evident in mice from the early treatment group (Fig. 4*d*).

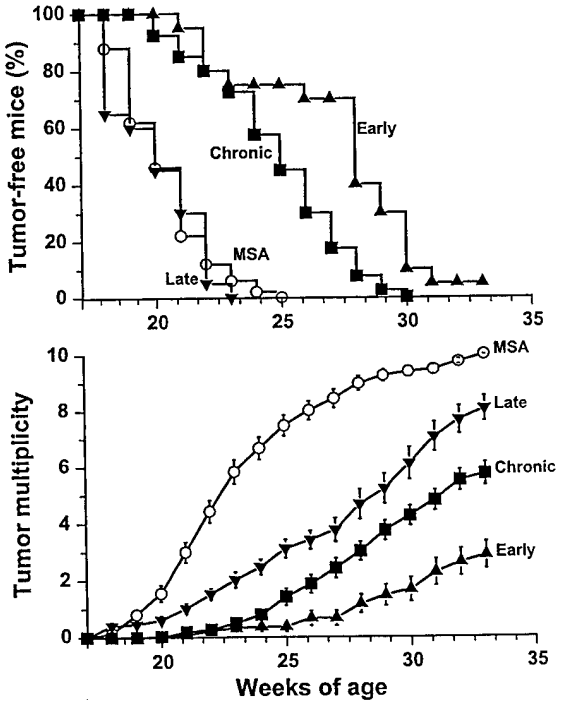


Fig. 3. Progression of mammary carcinogenesis in BALB-neuT mice receiving chronic, late, or early administration of IL-12. The percentage of tumor-free mice (*top panel*) and the mean number of palpable mammary carcinomas/mouse (calculated as the cumulative number of incident tumors/total number of mice; *bottom panel*) are shown. Fifty mice were MSA controls. There were 40 mice in the chronic treatment group, and 20 mice in both the early and the late treatment groups. Statistical analysis in the *top panel* shows that both early and chronic curves are significantly different (at least $P < 0.0005$ Mantel-Haenszel test) from the MSA curve, whereas the late curve is not significantly different. After week 21, all values in the *bottom panel* of early, chronic, and late treatment groups are significantly different from the corresponding values of the MSA group at least, ($P < 0.05$ Student's t test).

Inhibition of Tumor Vasculature in BALB-neuT Mice. This IL-12-induced delay of carcinogenesis closely fits the inhibition of tumor angiogenesis as assessed by direct microvessel count (Table 1). At 15 weeks, mammary glands from the MSA controls displayed vigorous capillary sprouts inside the atypical hyperplastic areas, whereas only a few capillaries surrounded the foci of *in situ* carcinoma. Minor vascular damage and inhibition of angiogenesis were evident in mice from the late treatment group. In contrast, a defective vascular network and a moderate reduction and a marked reduction of the number of microvessels were evident in mice from the chronic and early treatment groups (Fig. 2,*a–c*). These differences diminished markedly at the 25th week, when evident tumors were present in all treatment groups (Table 1; Fig. 2, *d–f*).

Proliferative Rate of BALB-neuT Tumors. To evaluate whether IL-12 treatments affect the growth rate of evident tumors, the time required by a tumor with a mean diameter of 4 mm to reach 8 mm in mean diameter was calculated for the first tumor in each mouse. IL-12 increased tumor doubling time, but this increase was too small to be significant. PCNA immunostaining to assess the rate of epithelial cell proliferation was mainly detected in the peripheral cell layer of neoplastic lobules in untreated mice and in all treatment groups. Evaluation of PCNA-positive cells, also failed to disclose appreciable differences among the treatments (Table 1).

Efficacy of Lower IL-12 Doses in BALB-neuT Mice. Because IL-12 appears to effectively inhibit the progression of HER2/*neu* carcinogenesis, the dose range in which such an inhibition is achieved was evaluated. When early treatment was performed using 10 and 50 times lower doses of IL-12, no delay in the appearance of the first tumor or reduction of the number of mammary glands with a palpable tumor was found, but a slight delay in tumor onset was seen (Fig. 5).

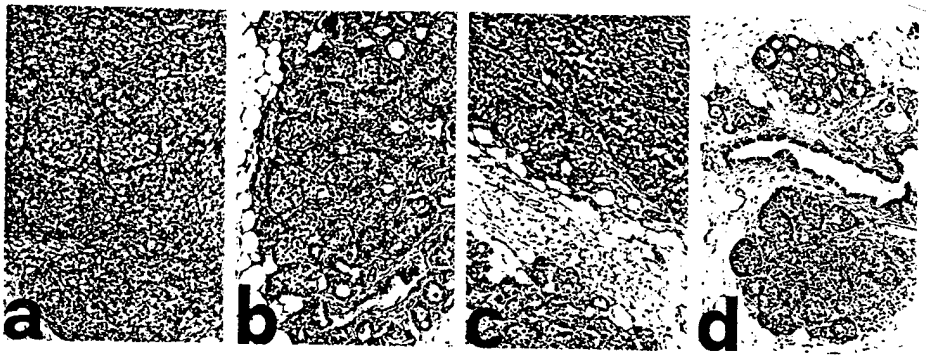


Fig. 4. Histopathology of mammary lesions in 25-week-old BALB-neuT mice. Invasive carcinomas formed by a uniform population of round cells grouped in alveolar structures are evident in the mammary glands of MSA controls (*a*). Multiple foci of carcinoma *in situ* associated with some hyperplastic islets were the main feature in mice from the chronic treatment group (*b*), whereas both invasive carcinomas and large carcinoma *in situ* were present in mice from the late treatment group (*c*). A restrained hyperplasia and a few foci of carcinoma *in situ* are evident in mice from the early treatment group (*d*).

Table 1 *Microvessel counts, expression of PCNA, and tumor doubling time in mammary tumors of BALB-neuT mice treated with IL-12*

	IL-12 treatment			
	MSA only	Chronic treatment	Late treatment	Early treatment
Microvessel count[a]				
15th week	22 ± 3	13 ± 2[b]	19 ± 3	9 ± 2c[b]
25th week	13 ± 2	12 ± 2	13 ± 4	11 ± 2
% of PCNA immunoreactivity				
30th week[c]	23 ± 6	21 ± 5	27 ± 9	22 ± 4
Doubling time of the diameter (4–8 mm) of the first mammary tumor	8 ± 5	12 ± 8	25 ± 19	25 ± 18

[a] Performed on cryostat sections with antiendothelial (CD31) monoclonal antibody. At least 10 fields/sample were counted. Values are expressed as mean ± SD of five 15- and 25-week-old mice.

[b] Values are significantly different ($P > 0.001$) from those of MSA controls.

[c] Performed on paraffin-embedded tissue sections with anti-PCNA monoclonal antibody.

Prevention of Carcinogenesis in FVB-neuN Mice. In FVB-neuN mice, the overexpressed *neu* proto-oncogene induces mammary carcinomas with a much longer latency time. Until the 22nd week, the mammary glands of these mice are histologically normal, whereas foci of atypical hyperplasia and carcinoma *in situ* become evident in a few glands of 25-week-old mice. Randomly, a few of them progress slowly toward invasive carcinoma, and a mean number of 2.5 tumors/mouse is evident at the 60th week. The 6-week-old and 22-week-old IL-12 treatments began when FVB-neuN mice were still free from macroscopic or microscopic mammary lesions (17). Both treatments significantly reduced tumor incidence and multiplicity as compared with MSA controls (Fig. 6). In contrast, 28-week-old treatment was almost ineffective. It began when focal hyperplasia and carcinoma *in situ* were already a common finding.

DISCUSSION

With distinct kinetics, transgenic female mice carrying the activated (BALB-neuT) or the proto-oncogene (FVB-neuN) rat HER2/*neu* under the MMTV promoter progress toward a consistent pattern of spontaneous mammary carcinogenesis that recapitulates a few features of the development of human mammary carcinoma (16). In both types of mice, IL-12 delays the onset and counteracts the multiplicity of mammary carcinomas. The present findings extend and confirm previous observations of mice treated with IL-12 during the whole progression of mammary carcinogenesis (16). Noguchi *et al.* (15) have shown previously that a similar IL-12 treatment also inhibits chemical carcinogenesis in mice.

Because these findings suggest that administration of IL-12 is of significance in hampering the progression of preneoplastic lesions, the specific issue addressed here was to define the stage of tumor progression in which these mechanisms are most effective. Should IL-12 administration be proposed as a preventive measure in healthy individuals with genetic risk of cancer patients, or can it also be of benefit once overt preneoplastic lesions are diagnosed? This is a significant question because genetic screening programs are singling out healthy individuals with genetic risk of cancer (18), and early diagnosis programs are detecting preneoplastic lesions (19).

As a result of the activated *neu* transgene, BALB-neuT mice display mammary cell atypia virtually from birth. The efficacy of IL-12 treatments in these mice suggests that the evolution of the tumor:host angiogenic relationship, rather than the intrinsic proliferative properties of transformed mammary cells, is the point of no return for IL-12 activity. In effect, the present findings suggest that at least part of this activity is due to the ability of IL-12 to inhibit the angiogenesis associated with mammary hyperplasia.

Around the 2nd week, almost all mammary glands of BALB-neuT mice display multiple foci of ductular atypical hyperplasia. Between

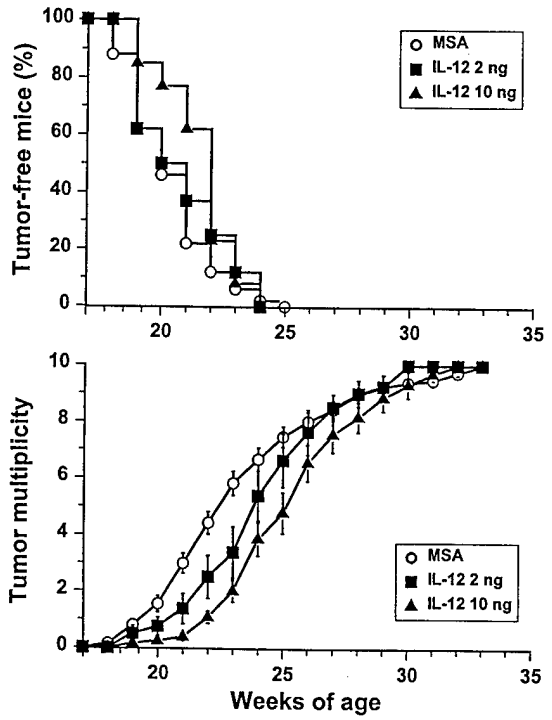


Fig. 5. Progression of mammary carcinogenesis in BALB-neuT mice from the early treatment group that received daily i.p. injections of 10 and 2 ng of IL-12. The percentage of tumor-free mice (*top panel*) and the mean number of palpable mammary carcinomas/mouse calculated as the cumulative number of incident tumors/total number of mice; (*bottom panel*) are shown. Each group consists of 10 mice. In the *top panel*, both IL-12 curves were not significantly different from the MSA curve by the Mantel-Haenszel test. Values of the groups receiving 10 and 2 ng of IL-12 are significantly different ($P > 0.001$) from corresponding values in mice of the same treatment group receiving 50 (first course) and 100 (following courses) ng of IL-12 (Fig. 2).

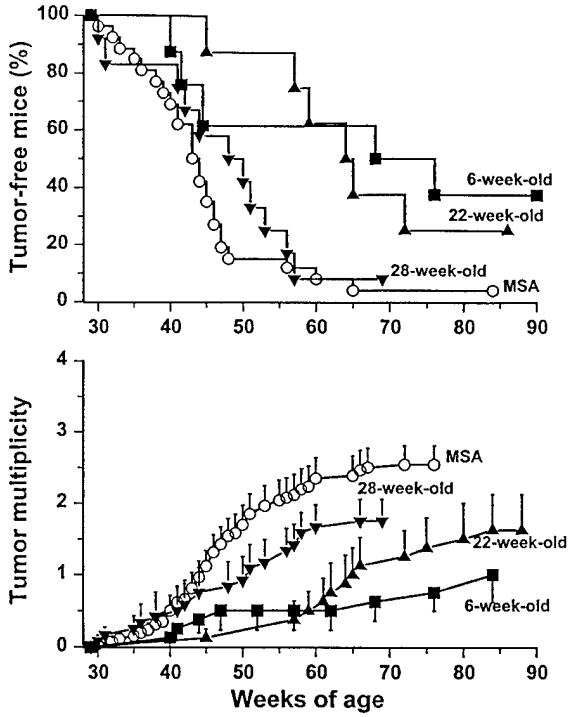


Fig. 6. Progression of mammary carcinogenesis in FVB-neuN mice receiving IL-12 treatment starting at 6 (6-week-old), 22 (22-week-old), or 28 (28-week-old) weeks of age. The percentage of tumor-free mice (*top panel*) and the mean number of palpable mammary carcinomas/mouse calculated as the cumulative number of incident tumors/total number of mice (*bottom panel*) are shown. Twenty-six mice were in the MSA control group, 12 mice were in the 28-week-old group, and 8 mice were in both the 6-week-old and the 22-week-old groups. In the *top panel*, both 6-week-old and 22-week-old curves were significantly different at least, ($P < 0.025$ Mantel-Haenszel test) from the MSA curve, whereas the 28-week-old curve was not statistically different from the MSA curve. In the *bottom panel*, after week 48, all values of both 6-week-old and 22-week-old groups were significantly different ($P < 0.05$, Student's t test) from the corresponding values of the MSA treatment group. No value of the 28-week-old curve was significantly different from the corresponding value of the MSA treatment group.

the 13th and 17th weeks, hyperplasia progresses to *in situ* carcinoma (Ref. 16; present study). Immunohistochemical staining with anti-CD31 monoclonal antibody shows that rich microvascularization inside preneoplastic lesions corresponds with their progression toward carcinoma, as shown in other tumor systems (23). This progression phase appears to be particularly appropriate for an angiostatic intervention (24, 25). Indeed, the most significant delay in tumor onset and progression is observed with the early treatment, when IL-12 courses given from the 2nd to the 14th week induced both a scanty vascularization and poorly developed hyperplastic foci.

The importance of the timing of IL-12 administration was further assessed with FVB-neuN mice, in whom an overexpressed *neu* proto-oncogene induces mammary carcinomas after a markedly longer latency. The 6-week-old treatment consists of a lifetime administration of IL-12 and is conceptually similar to the chronic treatment of BALB-neuT mice. Although the first course was markedly delayed on the 22-week-old treatment, it still started before an evident spreading of preneoplastic lesions. Both treatment schedules delay the onset of carcinomas and their multiplication. The period between the 22nd and the 28th week appears to be of critical importance because the 28-week-old protocol confers only a negligible protection. During these 6 weeks, in fact, normal mammary glands progress toward atypical hyperplasia and then toward carcinoma *in situ* and invasive carcinoma. Palpable tumors are first detected at 30 weeks.

The equivalent results from BALB-neuT and FVB-neuN mice suggest that IL-12 effectively inhibits mammary carcinogenesis when its administration accompanies the angiogenic switch. Its antiangiogenic effect appears to rest on the increased serum levels of IFN-γ and tumor necrosis factor-α released by activated T lymphocytes and natural killer cells (5, 7). The antiangiogenic (4, 8) and angiotoxic (26) activity of these two cytokines is stronger on those fragile capillary sprouts, which go with the shift from the preneoplastic to the neoplastic condition. Downstream mediators elicited by IL-12 may also act on neoplastic cells, in which they down-regulate the production of proangiogenic molecules (7, 27) and up-regulate the release of antiangiogenic factors such as IFN-inducible protein 10 and monokine induced by IFN-γ (7, 14). After the transition from hyperplasia to *in situ* and invasive carcinoma, capillary sprouting becomes restrained. The poor efficacy of late treatment in both BALB-neuT and FVB-neuN mice may depend on the lower sensitivity of mature and differentiated blood vessels of the more advanced neoplastic lesions to IL-12-induced angiostasis.

The decreased number of microvessels per microscopic field in both *in situ* and invasive carcinoma in comparison to hyperplastic areas suggests that this type of carcinoma, once developed, no longer requires a profuse vascular supply. The few vessels of the stroma of neoplastic lobular-alveolar structures are enough to sustain their relatively low rate of proliferation. In contrast, blood supply is a critical factor for most fast-growing transplantable tumors, even during their later stages. This necessity may account for the high efficacy of IL-12 against these tumors, even when they are large (3, 7). With tumors that progress slowly, antiangiogenic activity is only efficacious in specific progression stages (24). This narrow window of activity might account for the ineffectiveness of IL-12 in the management of human cancer, because only patients bearing advanced tumors are enrolled in clinical trials (28).

The antitumor action of IL-12 is not confined to its indirect influence on endothelial cells. Directly or through secondary cytokines, its triggers lytic activity and mediator release in a variety of tumor-infiltrating leukocytes, thus offsetting the continuous generation of new transformed cells (7, 10–12). The efficacy of the hampering of tumor progression by IL-12 probably rests on the sum of its activities and not simply on the blocking of tumor neoangiogenensis, as important as this may be. In effect, further subdivision of the early protocol in shorter treatment periods markedly reduced IL-12 efficacy (data not shown).

The lower efficacy of chronic *versus* early treatment could indicate that continuous IL-12 administration is suppressive (29), although this possibility is not endorsed by the results in FVB-neuN mice. It should be noted that from the second course, BALB-neuT and FVB-neuN mice received 100 ng/day IL-12 (*i.e.*, around 4.5–7.7 μg/kg). This dose is well tolerated, and almost no side effects were manifested (7, 16). It is probably close to the optimal active dose, because a 10- or 20-fold reduction abolishes its activity.

In conclusion, our data suggest that IL-12 effectively impairs the *neu* oncogene-driven progression of mammary carcinogenesis by interfering with the passage from atypical hyperplasia to invasive carcinoma. This interference appears to depend largely on indirect inhibition of tumor-associated angiogenesis. Its diminished efficacy in more advanced lesions and the dose range required pose some constraints on the use of IL-12 as an immunological alternative to current management of already manifest neoplastic lesions. Nevertheless, the efficacy of IL-12 points to enhancement of nonspecific immunity as an effective way to prevent mammary tumors in individuals at risk. Lifetime administration is not required for genetically determined cancers with a long natural history; instead, a precise definition of the carcinogenic events may allow preventive treatments starting relatively late in the life of individuals at risk.

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Appendix #4

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Publication of the International Union Against Cancer

p185^{neu} PROTEIN IS REQUIRED FOR TUMOR AND ANCHORAGE-INDEPENDENT GROWTH, NOT FOR CELL PROLIFERATION OF TRANSGENIC MAMMARY CARCINOMA

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Transgenic FVB-NeuN mice (N202) bearing the rat *neu* protooncogene driven by the mouse mammary tumor virus promoter/enhancer develop focal mammary carcinomas overexpressing the *neu*-encoded p185^{neu} protein. *In vitro* expression of p185^{neu} among mammary carcinoma cultures was heterogeneous, and we could establish some cell lines and clones displaying a complete loss of p185^{neu} expression, along with others with very high p185^{neu} protein level. Upon *in vivo* injection, p185^{neu}-positive cells gave rise to fast-growing tumors with a short latency, while p185^{neu}-negative cells required a very long latency time, and the resulting tumors were invariably p185^{neu}-positive. The lower growth ability of p185^{neu}-negative cells *in vivo* was also confirmed in athymic nude mice. *In vitro*, analysis of anchorage-independent growth in soft agar revealed colony formation from p185^{neu}-positive but not p185^{neu}-negative cells. The direct involvement of p185^{neu} in clonogenicity was demonstrated by the inhibition of p185^{neu}-positive colony growth in soft agar in the presence of an anti-p185^{neu} monoclonal antibody. By contrast, a higher level of anchorage-dependent clonogenic growth and proliferation was observed in p185^{neu}-negative cells as compared to p185^{neu}-positive cells, thus explaining the relative ease with which p185^{neu}-negative cell lines and clones were established *in vitro*. Together, the results indicate that p185^{neu} expression can lead to tumor formation and metastasis through the modification of intrinsic properties of cells related to anchorage-independent growth ability rather than to proliferation or host-dependent mechanisms.
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The rat *neu* protooncogene and its human homologue *HER-2* encode a 185 kDa transmembrane glycoprotein (p185$^{HER-2/neu}$) with intrinsic tyrosine kinase activity related to the epidermal growth factor receptor (King *et al.*, 1985; Coussens *et al.*, 1985). Amplification and overexpression of *HER-2* has been implicated in the pathogenesis of several human malignancies, including breast, ovarian and lung carcinomas (Slamon *et al.*, 1989; Kern *et al.*, 1994; Felip *et al.*, 1995), and its overexpression correlates significantly with poor prognosis in subsets of patients with breast cancer (Slamon *et al.*, 1987; Rilke *et al.*, 1991; Pupa *et al.*, 1996). *In vitro* studies using rodent cells indicated that p185^{neu} overexpression *per se* is sufficient to induce malignant transformation (Di Fiore *et al.*, 1987). In several strains of transgenic mice carrying the activated rat *neu* oncogene under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter/enhancer, early onset of transgene expression in the mammary epithelium of female and male mice results in the synchronous appearance of tumors involving all mammary glands (Muller *et al.*, 1988). FVB-NeuN mice (line N202) overexpressing the unactivated *neu* transgene driven by the MMTV promoter/enhancer develop focal mammary tumors, but only in females and with a long latency (Guy *et al.*, 1992). Together, these findings identify *HER-2/neu* as a potent oncogene. However, there is no direct evidence to indicate a causal relationship between p185^{HER-2} overexpression and malignancy, and the prognostic value of p185^{HER-2} overexpression *per se* is still controversial (Toikkanen *et al.*, 1992; Seshadri *et al.*, 1993; Hartmann *et al.*, 1994; Schonborn *et al.*, 1994; Revillion *et al.*, 1998). Clinical and molecular data indicate a much lower incidence of *HER-2* gene amplifications in distant metastases than in primary tumors (Driouch *et al.*, 1997), and the risk of metastasis is actually higher in breast cancer patients with p185^{HER-2} underexpressing primary tumor than in patients with normal or overexpressed p185^{HER-2} protein (Koscielny *et al.*, 1998).

The role of *HER-2/neu* gene in tumor transformation and progression is still unclear. Some experimental data suggest that *HER-2/neu* acts on proliferation since the signaling pathway of p185$^{HER-2/neu}$ involves MAP kinase activation; indeed, a very strong association between p185^{HER-2} overexpression and high number of mitoses has been reported in human breast carcinomas (Rilke *et al.*, 1991). However, other data suggest a role for *HER-2* gene in metastatic potential or in induction of hormone independence in a manner unrelated to cell proliferation (Yu *et al.*, 1994; Tan *et al.*, 1997). Furthermore, the dual role of *HER-2* activation in proliferation or differentiation, depending on the cell type has been demonstrated either by transfection of the *HER-2* gene or by treatment of cells with anti-p185^{HER-2} antibodies (Bacus *et al.*, 1992; Peles *et al.*, 1993; Giani *et al.*, 1998).

To investigate the role of *HER-2/neu* overexpression in tumorigenesis, we used the FVB-NeuN mouse strain (N202), which is transgenic for the rat *neu* protooncogene (Guy *et al.*, 1992) and represents a faithful model of human tumors overexpressing the p185^{HER-2} oncoprotein. We found that a complete loss of p185^{neu} oncoprotein expression is not uncommon among cells derived from transgenic mammary carcinomas, and we took advantage of this model to investigate the properties of interconverting p185^{neu}-positive and p185^{neu}-negative carcinoma cells. Our results indicate that the p185^{neu} oncoprotein does not contribute to the unrestricted proliferation of mammary carcinoma cells but is indispensable for the clonogenic anchorage-independent growth that underlies the ability to form progressive tumors *in vivo*.

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MATERIAL AND METHODS

Transgenic mice and spontaneous mammary carcinomas

A colony of FVB-neuN mice, transgenic line N202 (Guy *et al.,* 1992; Boggio *et al.,* 1998), was established in our animal facilities from breeding pairs obtained from Dr. W.J. Muller, McMaster University, Hamilton, Ontario, Canada. Transgenic line N202 was derived by microinjection into the pronuclei of FVB/N fertilized one-cell zygotes of the *SphI-EcoRI* fragment excised from the plasmid pMMTVneuN, containing the *HindIII-EcoRI* fragment encoding the unactivated rat neu cDNA and SV40 polyadenylation and splicing signals from pSV2neuN (Guy *et al.,* 1992). Both virgin and breeder females of this transgenic line develop spontaneous mammary carcinomas that give rise to distant lung metastases (Guy *et al.,* 1992; Boggio *et al.,* 1998). Mice were maintained under strict inbreeding conditions. The presence of the rat *neu* transgene was routinely checked by polymerase chain reaction (PCR) on tail DNA using primers hybridizing to vector (5′-ATCGGTGATGTCGGCGATAT-3′) and to MMTV sequences (5′-GTAACACAGGCAGATGTAGG-3′). Female mice developed mammary carcinomas with a mean latency time of about 40 weeks. Tumors were either dissociated enzymatically for cytometric analysis of p185^{neu} expression or processed for morphologic analysis (Lollini *et al.,* 1998).

Morphologic analysis

For histologic evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin-eosin. For immunohistochemistry, acetone-fixed cryostat sections were incubated for 30 min with a goat polyclonal IgG recognizing rat p185^{neu} (C-18, Santa Cruz Biotechnology, Santa Cruz, CA), washed and overlaid with biotinylated anti-goat IgG (Vector, Burlingame, CA) for 30 min. After washing to remove unbound Ig, slides were incubated with avidin biotin complex/alkaline phosphatases (Dako, Glostrup, Denmark).

Enzymatic dissociation of tumors

Tumor samples were freed from hemorrhagic and necrotic parts, washed in phosphate-buffered saline (PBS), finely minced with scissors and digested with a standard tissue culture grade trypsin-EDTA solution (0.5 mg/ml trypsin, 0.2 mg/ml EDTA, Life Technologies, Milan, Italy) at 37°C for 15 min; dissociated cells were washed twice in PBS and counted in a hemocytometer. Previous tests using cell cultures showed that this enzymatic treatment does not affect neu antigens.

Establishment of transgenic mammary carcinoma cell cultures

A series of cell lines and clones was established at Istituto di Cancerologia from transgenic mammary carcinomas. Tumor samples minced with scissors were seeded in tissue culture flasks in Dulbecco's modified minimal essential medium (DMEM) + 20% fetal bovine serum (FBS) (Life Technologies) and incubated at 37°C in a humidified 5% CO_2 atmosphere. Cultures were periodically washed briefly (1–2 min) with trypsin-EDTA to detach contaminating fibroblasts without damage to epithelial areas. When the epithelial monolayer reached confluency, usually 2–5 months after plating, cells were subcultured at low split ratios (usually 1:2). Established cell lines and clonal derivatives were routinely subcultured twice weekly at 1:4–1:8 split ratios.

Flow cytometry

The product of the transgene, rat *neu,* was detected using monoclonal antibody 7.16.4 (Oncogene Research Products, Cambridge, MA). Cells were stained in a standard indirect immunofluorescence procedure (De Giovanni *et al.,* 1991) with primary antibody followed by a fluorescein-conjugated anti-mouse IgG (Kirkegaard and Perry, Gaithersburg, MD). After final washings, cells were resuspended in PBS containing 1 μg/ml of ethidium bromide to gate out dead cells, and analyzed by FACScan flow cytometry (Becton Dickinson, Mountain View, CA). Tumor samples were analyzed after gating for cell dimension (forward scatter) and granularity (side scatter) to exclude debris, passenger leukocytes and erythrocytes.

Tumorigenicity and metastasis studies

Healthy young (8–16 weeks old) female transgenic mice or 5-week-old *nu/nu* female mice on Swiss CD-1 background (Charles River Laboratories, Calco, Italy) were used for the analysis of tumorigenicity and metastatic ability of cultured cells. Tumors were induced by injecting mice subcutaneously (s.c.) in the right inguinal region with 0.2 ml of a single-cell suspension containing 10^6 viable cells. Tumor incidence and growth were evaluated twice weekly. Neoplastic masses were measured with calipers; tumor volume was calculated as $\pi/6 \cdot [\sqrt{(a \cdot b)}]^3$ in which a and b are two perpendicular major diameters. Experimental metastases were evaluated 30 days after the injection of 10^5 cells in a lateral tail vein. Lung nodules were contrasted with black India ink; metastases were counted in dissected lung lobes under a stereoscopic microscope.

Molecular analysis of rat neu *gene presence and expression*

For genomic DNA extraction, 0.5×10^6 cells were pelleted, resuspended in 0.2 ml of extraction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.45% Tween 20, 0.45% NP40, and 0.12 mg/ml proteinase K) and incubated at 56°C for 90 min. Proteinase was inactivated by treatment at 95°C for 30 min. PCR was performed on 1 μl of DNA in a final volume of 50 μl. Primers to amplify vector-MMTV sequences were as described above in "Transgenic mice." Primers to amplify rat *neu* sequence were: 5′-AGGGCAACTTGGAGCTTACCTACG-3′ and 5′-GGGTTCTGCCTGGGGTGGGA-3′; these primers amplify a 234 bp product from the rat *neu* transgene, whereas they do not amplify endogenous murine *neu* sequence.

Northern blot analysis

RNA was extracted with RNAzol™ B isolation solvent (Tel-Test, Inc., Friendswood, TX) following the supplier's instructions. RNA (20 μg/sample) was electrophoresed in a 1% agarose-formaldehyde gel, transferred to a nitrocellulose filter (Schleicher and Schuell, Keene, NH) and immobilized by UV-crosslinking. Hybridization was carried out using a [^{32}P]dCTP (Amersham) probe, obtained by BamHI digestion of *neu* cDNA corresponding to 2248 bp of 3′ end excised from pRNeucDNA-H (kindly provided by Dr. P. Vezzoni), using a random-primed DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). After stripping, the membrane was hybridized with a control [^{32}P]dCTP β-actin probe (Oncogene Research Products).

Immunoprecipitation and Western blot analysis

Cells were trypsinized, washed twice with cold PBS and solubilized for 45 min on ice in lysis buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, and 0.5% Triton X-100) containing protease plus phosphatase inhibitors, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM sodium orthovanadate (Na_3VO_4). Cell lysates were cleared by centrifugation at 4°C for 10 min at 10,000 g. Protein concentration was determined by the BCA protein assay (Pierce Biochemical Co., St. Louis, MO). Cell lysates (1.5 mg protein/sample) were immunoprecipitated after preclearing for 30 min with GammaBind Plus Sepharose (Pharmacia Biotech, Uppsala, Sweden) by incubation on a rocker with 3 μg/ml of monoclonal antibody 7.16.4 (Oncogene Research Products) or with mouse myeloma NSO-conditioned culture medium as negative controls for 3 hr at 4°C. Sepharose was added (20 μl) and after 3 hr incubation, immune complexes were washed three times with lysis buffer, eluted and denaturated by heating for 5 min at 95°C in reducing Laemmli sample buffer and resolved in a 7.5% polyacrylamide gel. Separated proteins were electrophoretically transferred to nitrocellulose membrane (Hybond C, Amersham, Little Chalfont, UK) and incubated at room temperature for 1 hr with anti-phosphotyrosine monoclonal antibody 4G10 (1.5 μg/ml; Upstate Biotechnology, Inc., Lake Placid, NY) and rabbit polyclonal anti-p185^{neu} serum C-18 (2 μg/ml; Santa Cruz Biotechnology, Inc.) followed by incubation with anti-mouse Ig and/or anti-rabbit Ig horseradish peroxidase-linked whole antibodies (1:10,000)

(Amersham) and visualized using the ECL detection system (Amersham) according to the supplier's instructions.

Analysis of in vitro cell growth and proliferation

Anchorage-independent growth in agar was determined by suspending 10^4–2×10^5 cells in DMEM + 20% FBS containing 0.33% agar; cells suspensions were then layered on a 5 ml base of 0.5% agar in 60 mm Petri dishes. Colony growth was monitored twice weekly and determined by counting at low magnification 14 days after seeding. In some experiments, cells were seeded in the presence of 1 μg/ml of anti-p185^{neu} monoclonal antibody 7.16.4 or of an isotype-matched antibody of unrelated specificity. For anchorage-dependent clonogenicity, 200–6,400 cells were seeded in 60 mm tissue culture Petri dishes in DMEM + 20% FBS. After 14 days, colonies were fixed in methanol, stained with Giemsa and counted with an inverted microscope at low magnification. Growth on plastic was studied by seeding 5×10^5 viable cells in 25 cm^2 flasks. Growth curves were obtained by direct count of cells harvested with trypsin-EDTA for 5 days after seeding. To determine saturation cell density, cells were grown to confluency and medium was renewed every 1–2 days thereafter; microscopic and visual inspection of cultures was carried out daily to exclude cell losses due to detachment from substrate. Cell yield was repeatedly evaluated over successive time points.

RESULTS

Expression of p185^{neu} in spontaneous mammary carcinomas of neu transgenic mice

Cytofluorometric analysis of 53 independent mammary carcinomas from 19 individual FVB-NeuN transgenic mice revealed consistently high levels of p185^{neu} expression on the tumor cell membrane (Fig. 1*a*). A narrow distribution of p185^{neu} expression was observed within each tumor (Fig. 1*b*), and no bimodal peaks suggestive of p185^{neu} loss variants were resolved. Immunohistochemical analysis of tumor specimens with an anti-p185^{neu} antibody confirmed the high level expression of the transgene in the mammary carcinomas (Fig. 2*b*).

In vitro selection of p185^{neu}-negative cells

A cell line, designated N202.1, was derived from one mammary carcinoma. Cytofluorometric analysis indicated high level expression of p185^{neu} by these cells but considerable heterogeneity of p185^{neu} expression in a series of clones randomly derived from N202.1, i.e., clone N202.1E expressed no detectable surface p185^{neu}, and two other clones (N202.1B and N202.1C) showed low expression (Fig. 3, left). All clones, regardless of p185^{neu} expression, shared the selective loss of H-2D^q expression (data not shown) previously found in mammary carcinomas of N202 origin (Lollini *et al.*, 1998), thus confirming a common neoplastic origin.

To determine whether the decline of p185^{neu} expression in these clones was due to the cloning procedure, we analyzed a series of long-term cultures established from independent transgenic mammary carcinomas (Fig. 3, right). One, designated TT3, had no detectable p185^{neu} expression similar to clone N202.1E, while a second, TT5, resembled clones N202.1B and N202.1C in the low level of neu expression. PCR analysis of genomic DNA with primers specific for rat *neu* and for sequences present in the vector originally used to generate the transgenic mice indicated the presence of the transgene in all clones, independent of cell surface p185^{neu} expression levels (Fig. 4*a*). *neu* mRNA was not detected in p185^{neu}-negative cells (N202.1E and TT3) by Northern blot analysis (Fig. 4*b*), suggesting control of expression at the transcriptional level. Western blot analysis of immunocomplexes from N202.1E and TT3 lysates revealed no p185^{neu} oncoprotein expression (Fig. 4*c*). p185^{neu} protein and its tyrosine phosphorylation were detected in N202.1A cells and, to a lesser extent, in N202.1B but not in N202.1E or TT3 cells (Fig. 4*d*).

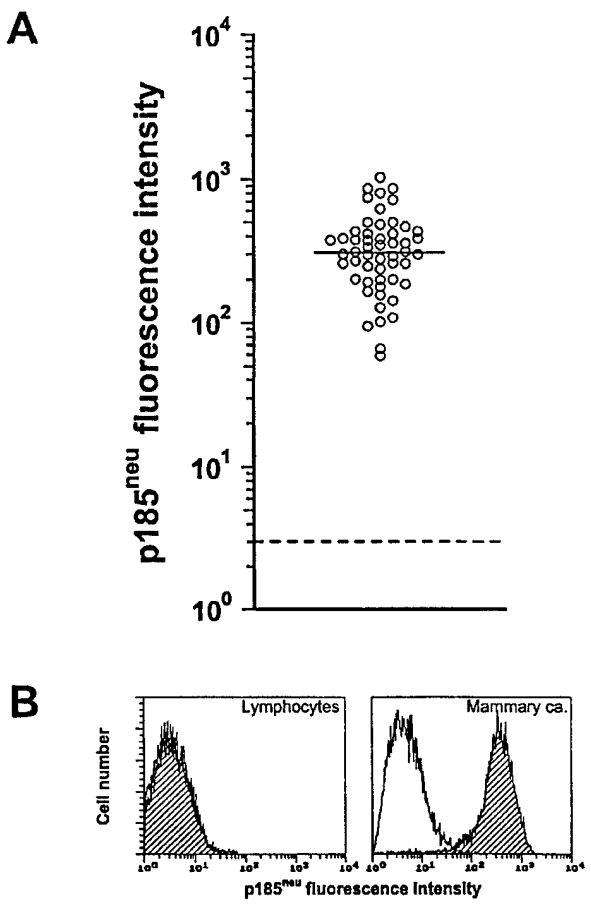

FIGURE 1 – Cytofluorometric analysis of p185^{neu} expression in enzymatically dissociated cells from mammary carcinomas of FVB-NeuN transgenic mice. *a:* Fluorescence intensity of p185^{neu} expression in 53 consecutive tumors. Continuous line represents the mean value of p185^{neu} fluorescence intensities. Dashed line represents the mean value of controls treated with secondary antibody alone. *b:* p185^{neu} expression in a representative mammary tumor (right) as compared with expression levels in normal T lymphocytes (left). Open curves: cells stained with secondary antibody alone; shaded curves: cells stained with anti-p185^{neu} antibody.

p185^{neu}-Negative cells give rise to dormant tumors

Comparison of the ability of p185^{neu}-positive and p185^{neu}-negative cells to grow as tumors in syngeneic transgenic mice indicated a very delayed onset of tumors generated from p185^{neu}-negative N202.1E and TT3 cells as compared with the short latency characteristic of p185^{neu}-positive N202.1A and N202.1B tumors (Fig. 5). Nevertheless, progressive tumors eventually appeared at the site of cell injection in almost all mice treated with N202.1E or TT3 cells, and the growth rate of established tumor masses was similar to that of N202.1A and N202.1B tumors (Fig. 5). Latency times showed by tumors induced by N202.1E or TT3 were almost always shorter than those observed for spontaneous tumors.

Similar analyses of tumorigenicity in athymic nude mice to exclude the interference of immune-mediated phenomena again revealed the pronounced delay in tumor appearance after p185^{neu}-negative cell injection as compared with p185^{neu}-positive cells: N202.1E-induced tumors appeared in 83% of nude mice with a latency time of 70–170 days, whereas all the mice receiving N202.1A cell injection became tumor-positive in 20–30 days.

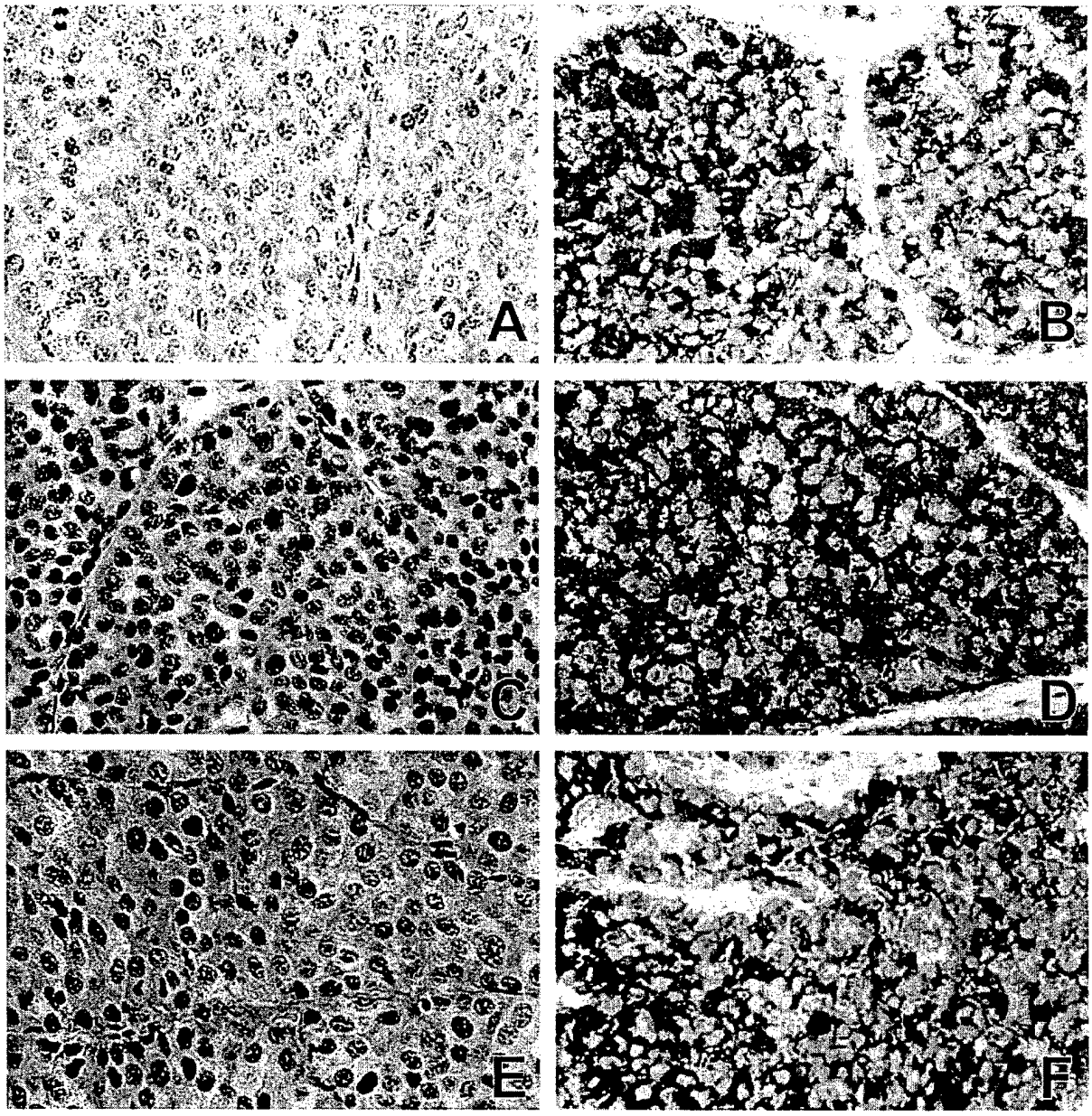

FIGURE 2 – Histological features (*a, c, e*) and immunohistochemical analysis for p185^{neu} expression (*b, d, f*) of mammary tumors grown in FVB-NeuN mice. *a,b:* spontaneous tumor; *c,d:* tumor induced by s.c. injection of N202.1A cells; *e,f:* long-latency tumor induced by s.c. injection of N202.1E cells. N202.1A and N202.1E cells both gave rise to a tumor morphologically similar to the spontaneous tumor. Anti-p185^{neu} immunostaining revealed an intense pattern of distribution of p185^{neu} expression in all tumors. *a,c,e*: hematoxylin-eosin, magnification: ×630; *b,d,f*: immunohistochemistry, magnification: ×630.

Experimental lung metastases were detected in all transgenic mice injected i.v. with N202.1A (median number of lung nodules = 76, range 60–111), whereas N202.1E cell-injected mice never developed metastases.

p185^{neu}-Negative cells induce p185^{neu}-positive tumors

The morphological features of N202.1E tumors were highly reminiscent of those found in primary tumors or in fast-growing tumors induced by p185^{neu}-positive N202.1A cells (cf. Fig. 2*e* with *a* and *c*). Immunohistochemical analysis of long-latency tumors arising after injection of p185^{neu}-negative N202.1E cells revealed a uniformly high expression of p185^{neu} (Fig. 2*f*). The intensity and cellular distribution of p185^{neu} staining were similar to those found in spontaneous primary tumors (cf. Fig. 2*f* with 2*b*) and slightly less homogeneous than that obtained with fast-growing p185^{neu}-positive N202.1A clone (cf. Fig. 2*f* with 2*d*).

Cytofluorometric analyses of tumor cells freshly dissociated from three mammary carcinomas produced by s.c. injection of clone N202.1E revealed in each case cells expressing high levels of p185^{neu} (Fig. 6). Long-term cell cultures that maintained high p185^{neu} expression over several *in vitro* passages were derived

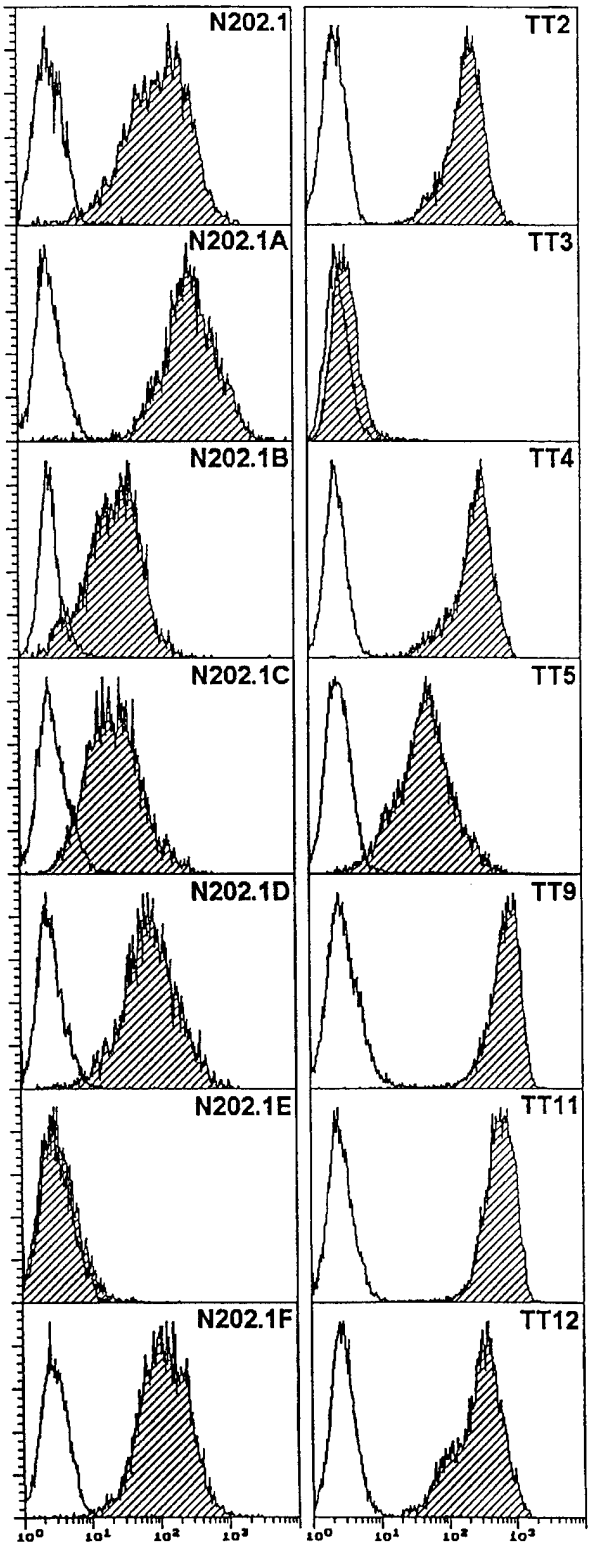

FIGURE 3 – Heterogeneous $p185^{neu}$ expression in vitro among different established cell lines and clones derived from mammary tumors of FVB-NeuN mice. *Left:* N202.1 cell line and its clonal derivatives. *Right:* Cell cultures from independent mammary carcinomas. Open curves: cells stained with secondary antibody alone; shaded curves: cells stained with anti-$p185^{neu}$ antibody.

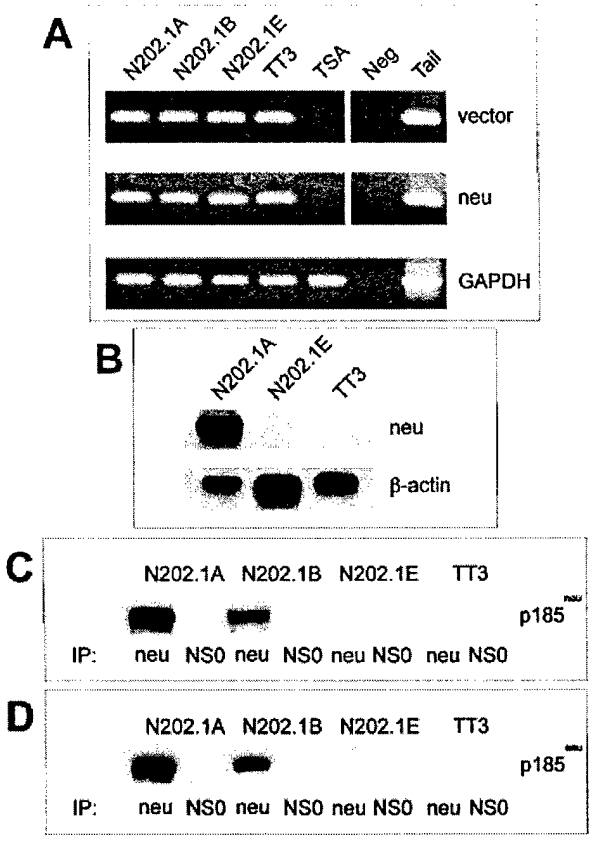

FIGURE 4 – Molecular analysis of *neu* transgene presence and expression in mammary carcinoma cell lines derived from FVB-NeuN mice. *a:* PCR analysis of genomic DNA from representative clones with high (N202.1A), low (N202.1B) or undetectable (N202.1E and TT3) cell surface $p185^{neu}$ expression. Mammary carcinoma cell line TSA of BALB/c origin was included to show the species-specificity of rat *neu* primers, that do not amplify endogenous murine *neu* sequences. Controls: neg, no template; tail, positive control DNA extracted from tail of transgenic FVB-NeuN mice. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Amplified products: vector = 219 bp; neu = 234 bp; GAPDH = 452 bp. *b:* Northern blot analysis of *neu* expression. *c:* Western blot analysis of cell extracts immunoprecipitated with anti-rat $p185^{neu}$ monoclonal antibody (neu) or with mouse myeloma NSO-conditioned culture medium as negative control. *d:* Immunoblot analysis of $p185^{neu}$ phosphorylation in cell extracts immunoprecipitated with anti-rat $p185^{neu}$ monoclonal antibody (neu) or with mouse myeloma NSO-conditioned culture medium as negative control.

from two such tumors. One of the cultures, designated 1E-neu+, was used for s.c. injection in syngeneic transgenic mice; tumors arose with a short latency time, unlike those observed after N202.1E injection (Fig. 5).

$p185^{neu}$ Expression favors anchorage-independent growth but hampers anchorage-dependent growth

Current tumorigenicity data suggest that $p185^{neu}$ expression confers a growth advantage to mammary carcinoma cells that could be mediated either by host-dependent interactions (*e.g.*, neoangiogenesis) or by intrinsic growth properties of tumor cells. To investigate this issue, we compared the *in vitro* growth of $p185^{neu}$-positive and $p185^{neu}$-negative mammary carcinoma cells.

Anchorage-independent clonogenicity was first analysed. N202.1A and 1E-neu+ cells were able to form large colonies in soft agar, while N202.1E produced no colonies at any cell concentrations tested, and TT3 cells formed only rare small clusters

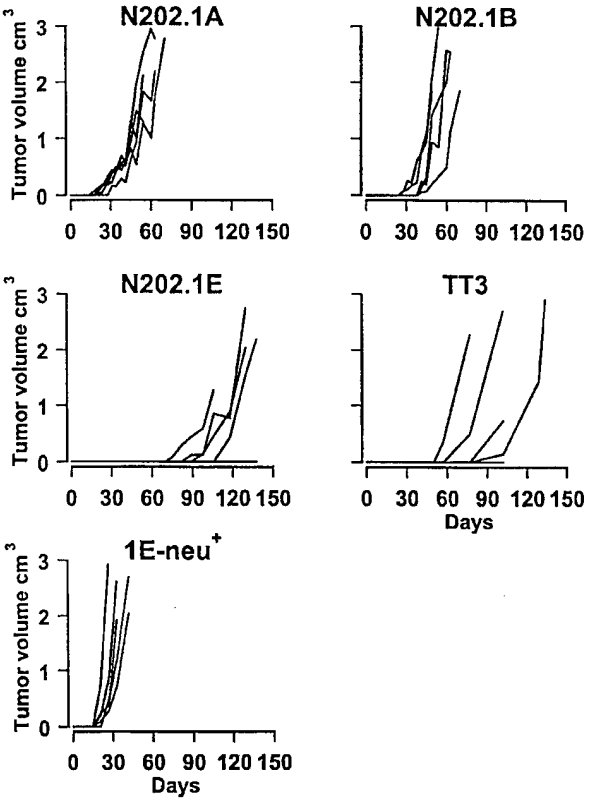

FIGURE 5 – Tumor growth of different transgenic mammary carcinoma cell lines injected s.c. into syngeneic FVB-NeuN mice. Tumor volumes are shown for each of 5 mice per group. Data are representative of at least 2 independent experiments.

(Figs. 7*a*, 8). Moreover N202.1A growth in agar was significantly inhibited in the presence of an anti-p185^{neu} monoclonal antibody but not of an irrelevant antibody (Fig. 7*b*).

On the contrary, anchorage-dependent growth of p185^{neu}-negative N202.1E and TT3 cells was significantly more robust than that of p185^{neu}-positive N202.1A and 1E-neu+ (Fig. 7*a*), explaining the relative ease with which p185^{neu} loss variants emerged during *in vitro* culture. The better adherent growth of p185^{neu}-negative cells was not determined by a different adhesion ability, as evaluated 24 hr after cell seeding (data not shown) but resulted from a higher clonogenic growth on plastic surfaces (Fig. 7*a*) and from higher proliferation rates, as also revealed by bromodeoxyuridine labeling (data not shown). However, p185^{neu}-positive cells continued their growth after confluency and reached saturation densities higher than those of p185^{neu}-negative cells (Fig. 7*a*). This fact could be related to a more differentiated pattern of growth with dome formation observed in adherent cultures of p185^{neu}-negative N202.1E and TT3 cells.

DISCUSSION

In the present study, we show that the oncogenic activity of p185^{neu} contributes to the transformed and tumorigenic phenotype, not to cell proliferation of transgenic mammary carcinomas. In fact, overexpression of p185^{neu} in our murine system appeared to increase anchorage-independent growth but negatively affect anchorage-dependent cell proliferation and clonogenicity. The better adherent growth of p185^{neu}-negative cells could be related to molecules involved in adhesion phenomena and down-regulated in p185^{neu}-positive cells such as galectin-3 (data not shown).

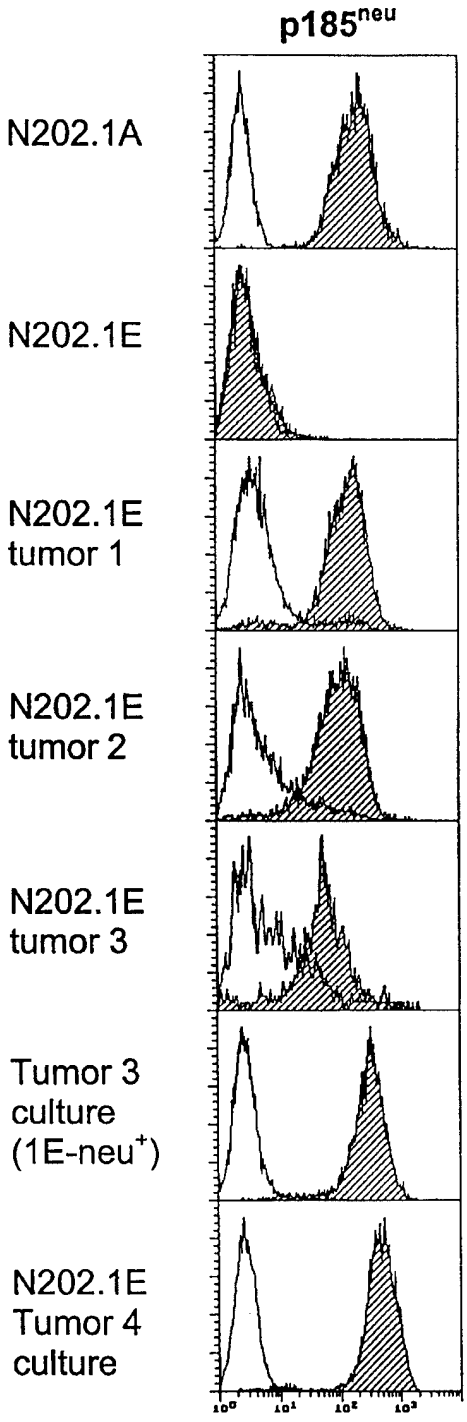

FIGURE 6 – Cytofluorometric analysis of p185^{neu} expression in long-latency tumors induced by s.c. injection of p185^{neu}-negative N202.1E cells and derived cultures. Open curves: cells stained with secondary antibody alone; shaded curves: cells stained with anti-p185^{neu} antibody.

Expression of p185^{neu} was apparently required for tumorigenicity and metastatic spread, since rapid tumor formation was observed only with p185^{neu}-positive cells, and all tumors that eventually arose from p185^{neu}-negative cells were invariably p185^{neu}-

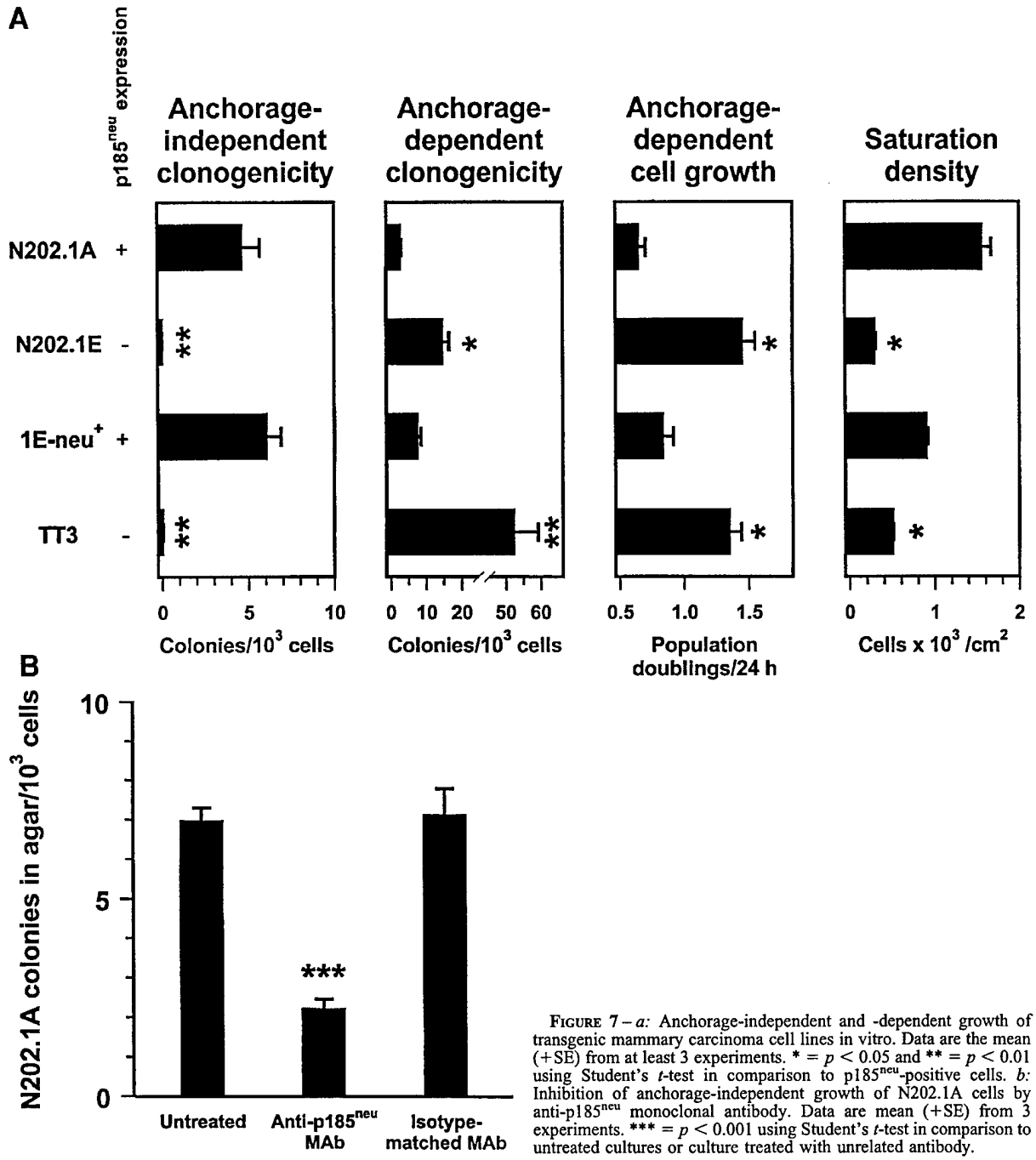

FIGURE 7 – *a:* Anchorage-independent and -dependent growth of transgenic mammary carcinoma cell lines in vitro. Data are the mean (+SE) from at least 3 experiments. * = $p < 0.05$ and ** = $p < 0.01$ using Student's *t*-test in comparison to p185^{neu}-positive cells. *b:* Inhibition of anchorage-independent growth of N202.1A cells by anti-p185^{neu} monoclonal antibody. Data are mean (+SE) from 3 experiments. *** = $p < 0.001$ using Student's *t*-test in comparison to untreated cultures or culture treated with unrelated antibody.

positive. *In vitro* studies revealed that p185^{neu}-dependent tumorigenicity depended on the ability to grow anchorage-independently and to reach high saturation densities, rather than on superior cell proliferation. It is well known that anchorage independence and growth to high densities are related to tumor formation by transformed cells, and our results are in good agreement with earlier findings obtained by gene transfection of *neu* into NIH3T3 fibroblasts (Baasner *et al.*, 1996; Di Fiore *et al.*, 1987), which acquire tumor-forming ability in parallel with *in vitro* growth properties similar to those controlled by p185^{neu} in our transgenic mammary carcinoma system.

In the context of normal mammary tissue, the growth autonomy conferred by p185^{neu} expression might be related to discrete developmental stages in which anchorage-independence and growth at high cell density are required. In pregnant rats, an increase in p185^{neu} expression was observed during the last steps of functional development of the normal mammary gland (Kokai *et al.*, 1987). In human prepartum and lactating mammary specimens, p185^{HER-2} expression was detected at an intensity comparable to that of breast carcinomas (Kacinski *et al.*, 1995). In another study, transfection of *HER-2* gene into MCF-7 breast carcinoma cells appeared to increase differentiation of these cells (Giani *et al.*, 1998).

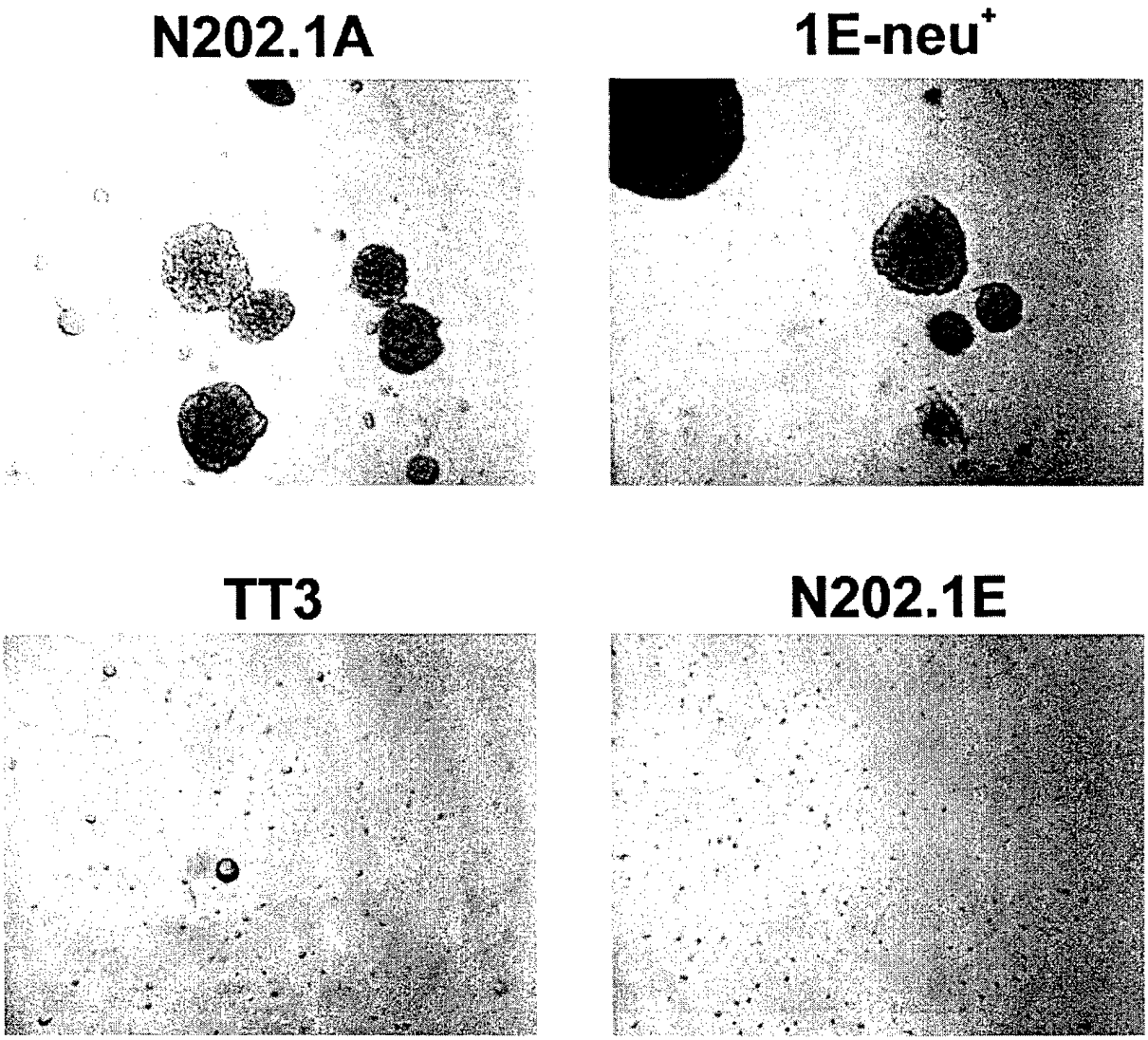

FIGURE 8 – Soft agar cultures of transgenic mammary carcinoma cell lines positive or negative for p185^{neu} expression. Magnification: ×25.

The pattern of tumor growth of p185^{neu}-negative cells, observed in our study, *i.e.*, a long latency period followed by the emergence of a fast-growing variant, is reminiscent of the behavior of dormant tumors (Demicheli *et al.*, 1994). Colony formation in soft agar was completely predictive of the ability to form rapidly growing tumors. The molecular events that determine dormancy and the restart of tumor growth in human breast carcinoma are not known but current hypotheses include mechanisms based on angiogenesis and interaction with the immune system (Uhr *et al.*, 1997). We suggest that changes in the level of p185$^{HER\text{-}2/neu}$ expression might be causally related to variations in the dormant status of breast carcinoma.

In conclusion, our results indicate that p185$^{HER\text{-}2/neu}$ expression is not required for the unrestricted proliferation of mammary carcinoma cells but is indispensable for specific steps of progression involving anchorage-independent growth of tumor cells, which determine the ability to form aggressive and metastatic tumors.

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Immunoprevention of Cancer: Is the Time Ripe?[1]

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Abstract

Immunotherapy applied to patients with established tumors rarely leads to an objective response, whereas patients apparently free from disease after conventional treatment and at risk of recurrence are beginning to receive vaccination. New classes of patients or not-yet patients are those with a high genetic or environmental risk of developing cancer. They may draw benefit from a "soft" treatment such as vaccination. This overview discusses the prospects of immune stimulation as a means of cancer prevention by inducing various forms of nonspecific or even specific immunity. Attainment of this goal provides the rationale and motivation for embarking on such a new and potentially rewarding enterprise.

Immunotherapy is emerging as an effective way to cure cancer (1–4) thanks to the dramatic progress that has led to the molecular and genetic definition of the tumor-host immune relationship. A detailed characterization of many tumor cell surface molecules that act as TAAs[3] is now available (5, 6). A second cornerstone has been provided by elucidation of the way in which TAA peptides are presented to T lymphocytes in association with glycoproteins of the MHC (7, 8) and the role of dendritic cells (9) and costimulatory (10), danger (11), and cytokine (12) signals. Genetic engineering of antibody molecules (13), soluble costimulatory signals (14–16), and tumor (17) and dendritic (18) cells is used to intensify the immune response and skew it toward Th1 or Th2 reactivity. This crucial information forms the groundwork for most ongoing immunotherapy clinical trials whose clinical setting is elicitation of an immune response in a tumor-bearing patient.

Determination of which kind of patients are eligible for Phase III clinical trials is not a minor issue (19, 20). Practical and ethical constraints result in the enrollment of advanced cancer patients in Phase I trials, whereas experimental mouse data suggest that the immunity induced by specific vaccination is much more effective in the inhibition of incipient tumors than in the cure of established tumors. Elicitation of a significant response in animals with advanced tumors is exceedingly difficult, and only a minority of tumor-bearing mice are cured (21). As a tumor increases in size, it becomes refractory to immunotherapy. Its genetic instability leads to the selection of antigenic variant clones (22, 23), whereas the characteristics of its stroma (24), the peculiarity of its blood vessels (25), and its release of immunosuppressive factors (26) build up a sort of privileged site proof against immune attack.

A similar picture is emerging from Phase I immunotherapy trials. Only a few patients with established tumors display objective and in any event temporary responses (2, 3). The immunological performance status of the patients enrolled is obviously suboptimal. Most have already been treated in various ways and no longer respond to conventional therapy. Their tumor cells are selected because of their ability to escape immune reactions, and their tumor masses can suppress an immune attack. At present, immunotherapy seeks to overcome these obstacles by aggressive or combined forms of treatment (21), whereas it is becoming evident that active immunotherapy is probably not a rational option in advanced cases. Indeed, repeated failures could even jeopardize the whole of what immunotherapy is endeavoring to achieve.

However, the lethality of a tumor usually stems from the relatively small number of its cells that remain after its surgical excision and are not killed by radiotherapy and chemotherapy. The importance of this issue lies in the experimental demonstration that active immunotherapy is effective against minimum residual disease and incipient metastases and in the control of tumor recurrences (27). Early immunotherapy after a successful conventional treatment is warranted. Clinical trials suggest that patients with minimal residual disease or patients expected to present recurrences after a long interval are those for whom immunotherapy may prove really effective because it induces a prolonged tumor free-survival, if not a complete cure (1, 4). Cancer vaccines tested as single agents in advanced melanoma patients are being tested in apparently disease-free patients in combination with chemotherapy. Significant results are expected from this more rational clinical approach (28). Once the efficacy of therapeutic immunization is demonstrated, it may also be proposed as an at-home or outpatient method for the elicitation of a long-lasting immunity after the conventional management of a small tumor (17).

The Prospects of Prevention

If immunotherapy is most effective in the early stages of tumor growth, consideration can be given to an even more radical view. The use of immunological measures to prevent or forestall cancer in healthy persons has not received much attention. This is surprising because most of the experimental data obtained with transplantable tumors show that new vaccines preimmunize mice against even a poorly or apparently nonimmunogenic tumor challenge (12, 29). Furthermore, the nonspecific immunity elicited by local and systemic cytokines effectively inhibits incipient tumors until they overcome a critical threshold and become clinically evident (27). Numerous and unambiguous experimental data show that the efficacy of both nonspecific and specific immunity declines as a tumor progresses (21, 27). Whether willfully or unthinkingly, however, the evidence from preimmunization tumor challenge experiments and the cytokine-induced collapse of incipient tumors is strained to demonstrate the efficacy of immunological measures in tumor therapy and not accepted for what it really says (30), namely, that immune reactivity possesses a great preventive potential, whereas its real therapeutic efficacy against established tumors is altogether another question (17).

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[3] The abbreviations used are: TAA, tumor-associated antigen; IL, interleukin; neuT, transforming HER-2/*neu* oncogene; neuN, nonmutated HER-2/*neu* proto-oncogene; Th, T helper.

Immunoprevention of cancer would have many advantages on its side. Healthy subjects, for example, may be expected to mount a more effective and powerful response than patients who have already been treated in various ways, whereas if the target tissue is still normal or displays no more than a localized preneoplastic lesion, the chances of success should be greater than when dealing with unresectable or disseminated tumors (31). Preneoplastic lesions do not yet display genetic instability, TAA mutations, and selection of the TAA-negative clones that characterize established tumors. They should also be more permeable to immune mechanisms because their cells do not markedly remodel the extracellular matrix or produce suppressive factors, and their vessel endothelium is not yet refractory to leukocyte extravasation (32–34). Several mutations in oncogene products are required for transformation. By contrast, an alerted immune patrol would detect the initial mutations and be ready to intervene before complete transformation takes place. Although antigen(s) associated with preneoplastic lesions (as well as those for many established neoplasms) have not been yet identified, the products of mutated oncogenes are probable candidates (35). Moreover, papillomas induced by methylcholanthrene are both antigenic and antigenically distinct from each other. This suggests that TAAs characterizing subsequent progressing sarcomas are already present at the preneoplastic stage (36).

Nonspecific Immunity in Cancer Prevention

The selection of not-yet patients and healthy individuals eligible for immunoprevention depends on the kind of treatment envisaged. Enhancement of nonspecific immunity and specific antitumor vaccination are two possible approaches. The advantage of a nonspecific antitumor response is that it can be applied directly to a broad range of individuals, irrespective of the type of TAA their foreseeable tumors may eventually express. However, it is not feasible to imagine healthy persons being treated nonspecifically for long periods. The results of the mouse experiments indicate that nonspecific stimulation should thus be restricted to not-yet patients with a genetic risk of cancer (34), individuals exposed to high carcinogen doses (37), patients with a preneoplastic lesion, and patients that probably have minimal residual disease after successful conventional treatment (27). Many not-yet patients with a high risk of cancer are, in fact, being recruited in ongoing programs to screen for preneoplastic lesions or gene mutations that predispose to cancer.

Women at risk for breast cancer or with preneoplastic lesions form a category for which nonspecific immunoprevention could be considered as a practical option.

However, the disclosure of a genetic risk of cancer and the presence of a preneoplastic lesion raise complex issues (38). Not a few individuals will find it difficult to cope with this information and may become deeply anxious about the possibility that they may have cancer. Routine cancer screenings, prophylactic mastectomy, and chemoprevention are all unpleasant and additionally stressful options (39, 40). A "soft" immunoprevention alternative would undoubtedly be welcome.

But what has nonspecific stimulation of immune reactivity to offer? A study of immunosurveillance against preneoplastic skin carcinomas suggested that it is not selective because elimination of such lesions was in no way related to their degree of malignancy (41). The extent to which nonspecific stimulation can prevent the onset of cancer in cases where a risk exists has been investigated by Noguchi *et al.* (37) in Dr. L. J. Old's laboratory. In their experiments, tumors were induced in BALB/c mice by s.c. injection of 3-methylcholanthrene. Delayed tumor appearance and reduced incidence were observed in mice receiving 100 ng of systemic IL-12 five days a week for 18 weeks (3 weeks on and 1 week off) during tumor latency. Secondary IFN-γ, IL-10, and tumor necrosis factor-α were evident in their sera. A high production of IFN-γ by CD8 T cells and a Th2$\rightarrow$Th1 or Th0 shift in the cytokine secretion profile of CD4 T cells were also noted.

The ability of similar doses of IL-12 to prevent tumors when administered to mice with a genetic risk of cancer was therefore studied by us (34) in two lines of transgenic mice expressing the rat HER-2/*neu* oncogene under the transcriptional control of mouse mammary tumor virus (34). Female BALB-neuT (H-2^d) mice carrying the transforming HER-2/*neu* oncogene show no morphological abnormalities of the mammary gland until 3 weeks of age. They then progress through atypical hyperplasia to *in situ* lobular carcinoma, and at 33 weeks of age, all 10 mammary glands display invasive carcinomas. In adult FVB-neuN (H-2^q) mice carrying the HER-2/*neu* proto-oncogene, neoplastic progression is less impetuous, as shown by a longer latency (38–49 weeks) and a lower tumor multiplicity (mean, 2.6 tumors/mouse). Treatment with IL-12 (five daily i.p. injections; 1 week on and 3 weeks off; the first course with 50 ng IL-12/day and the following courses with 100 ng IL-12/day) begun at 2 weeks of age in BALB-neuT mice and at the 21 weeks of age in FVB-neuN mice markedly delayed tumor onset and reduced tumor multiplicity. In both lines, tumor inhibition was associated with deficient peri- and intratumoral angiogenesis, infiltration of reactive cells, production of proinflammatory cytokines, and inducible nitric oxide synthetase activation.

We next set out to determine the stage at which administration of IL-12 is most effective. Was it simply a preventive measure in still healthy animals or could it also be of benefit once overt preneoplastic lesions are diagnosed? Groups of BALB-neuT and FVB-neuN mice received IL-12 at progressive times during carcinogenesis (42). In both lines, IL-12 was particularly effective in inhibiting the progression from hyperplasia to *in situ* and invasive carcinoma, *i.e.*, at the time of the angiogenic switch. Its antiangiogenic effect is markedly evident on the fragile capillaries sprouted during this switch. Late administration was poorly effective in both mouse lines, presumably because the mature and differentiated blood vessels of more advanced lesions are less sensitive to IL-12-induced inhibition. However, the antitumor action of IL-12 is not confined to its indirect influence on endothelial cells. Directly or through secondary cytokines, it triggers lytic activity and mediator release from a variety of tumor-infiltrating leukocytes and thus counters the continuous generation of transformed cells. Its efficacy, in fact, probably rests on the sum of its activities, and not simply on the blocking of tumor angiogenesis, important as this may well be (27). These experiments also show that lifelong administration is not required for genetically determined cancers with a long natural history. Precise definition of the carcinogenic events may allow preventive treatments to be performed only during a critical stage of the long carcinogenic progression.

The HER-2/*neu* oncogene is expressed in a substantial proportion of human mammary carcinomas. The close resemblance of the progression of mammary carcinogenesis in HER-2/*neu* transgenic mice to that in women suggests that the administration of nontoxic recombinant IL-12 regimens may be a significant prophylactic strategy. The direct proportionality between the length of carcinogenesis progression and the efficacy of IL-12 observed in these models suggests that stimulation of nonspecific immunity could be envisaged as an effective, preventive way of slowing human carcinomas (30).

The principles illustrated by these models are clear. The extent to which they reflect the situation in humans must obviously be established in clinical trials, especially because the immunological weight of IL-12 may not be the same in mouse and human tumors. In the meantime, further evidence that cytokine-elicited immunity can prevent tumor progression is provided by a randomized multicenter Phase III trial with low, nontoxic doses of IL-2 injected locally.

Patients with resectable $T_{2-4}N_{0-3}M$ squamous cell carcinomas of the head and neck receiving supplemental IL-2 before and after surgery displayed a significantly extended disease-free interval as compared with those treated only with conventional therapy.[4]

Specific Antitumor Vaccination of Persons at High Risk of Cancer

Specific vaccination of persons at risk and healthy individuals constitutes a very different scenario. Characterization of specific gene alterations or detection of preneoplastic lesions may indicate which organ and tissue are at risk. In a few cases, more precise information may show which oncogene product will probably be overexpressed or expressed in an altered form and allow vaccination against a single, specific TAA. Molecular characterization of altered gene products predictably destined to become TAAs will be the first step toward the engineering of selective vaccines (43). Otherwise, the patient should be vaccinated against the TAA most commonly expressed by the tumors foreseeable in a given organ.

Many new antitumor vaccines that induce an effective resistance to subsequent tumor challenge and inhibit minimal residual disease are already available (12, 29). The question of whether specific immunization can be successful once a cell population has been subjected to the initial carcinogenic hit has rarely been examined experimentally. However, it can be plausibly suggested that cytokines and more conventional adjuvants could induce an effective immune response against ignored or fully tolerated antigens. The specific immunity elicited in mice transgenic for rat Her-2/*neu* is a sign that specific vaccination induces strong immune responses against such antigens and may thus inhibit oncogenesis and extend survival (44–46).

General Antitumor Vaccination

One can also envisage the even wider application of antitumor vaccines to prevent tumors in the general population, as is done for infectious diseases. This point considers the possibility of preventing the onset of cancers related to an infectious agent by vaccination against the agent itself. This approach is applicable to a sizeable proportion of diverse human tumors including cervical carcinoma (human papillomaviruses), hepatocellular carcinoma (hepatitis B and C viruses), and Burkitt's lymphoma (EBV). A significant impact of hepatitis B vaccination on the incidence of hepatocellular carcinoma has already been reported (47), and promising results being obtained in preclinical models of papillomavirus oncogenesis (48) suggest that human vaccination will eventually be able to prevent cervical carcinoma (49).

Molecular and genetic data suggest that human TAAs identified as targets of CTLs (5) or by the SEREX technique (6) can be divided into classes. One class consists of tumor-specific antigens coded by genes expressed by tumors but not by normal cells, with the exception of male germinal cells. However, because these cells do not express MHC glycoproteins, they do not present peptides from the protein products of these genes on their surface. The use of these antigens in preventive vaccination is interesting because their number seems not to be endless, and they are shared by histologically distinct tumors arising in different organs. Furthermore, the telomerase catalytic subunit is markedly activated in more than 85% of human tumors, whereas it is silent in normal tissues and thus constitutes a sort of universal TAA (50). The second class of antigens derived from point mutations looks less interesting for general vaccination because they are unique for a given tumor, and their expression by a foreseeable tumor is poorly predictable. Nevertheless, in some cases, oncogenes and oncosuppressor genes display a narrow spectrum of mutations (*e.g.*, RAS; Ref. 51). In addition, chromosome translocations that give rise to fusion proteins display a relatively constant pattern of junction between the two genes.

Another class comprises antigens that are also expressed by normal cells of the same differentiation lineage. Immune reactions elicited against them could be coupled with the induction of an autoimmune disease. An additional class is formed of molecules expressed by normal cells and overexpressed by neoplastic cells. Here, too, there is a risk of inducing autoimmune reactions. In fact, once the immunological ignorance or tolerance against these antigens is overcome, effector mechanisms endowed with a lower threshold of activation may destroy both normal and neoplastic cells. However, experimental data from variously immunized mice did not disclose major autoimmune lesions as a side effect of vaccination with these antigens. On the contrary, a specific immune reaction often affected tumor cells overexpressing the target TAA and spared normal tissues where TAA was expressed at a much lower level (52).

Because many TAAs are shared by a variety of tumors, preventive immunization against most common human cancers with not many more than 20 TAAs would seem conceivable. A possible list would include the infectious agents mentioned earlier, mutated oncogenes, telomerase catalytic subunit, and antigen of the MAGE family. However, the erratic boundary between tumor immunity and autoimmunity (53) means that the risk of inducing an autoimmune disease is a major concern. This risk would be much weightier in the vaccination of healthy individuals as opposed to individuals at risk, where the scales of risk-benefit are markedly biased by the higher risk of cancer and the consequent shorter life expectancy. A further warning is related to "epitope spreading" (54). Several data in animal models show that immune responses to a few self-determinants shift drift and diversify with time and include other epitopes of the same proteins or other proteins.

The planning of vaccines à la carte by genetic engineering may be a way to selectively trigger reaction mechanisms that ignore cells that express a low density of the target antigens or are less prone to induce a widespread autoimmunity. Consideration must also be given to the balance between the kind of potential autoimmunity and the degree of lethality of the possible tumor. Autoimmune vitiligo, for example, would be a relatively small price to pay for protection against melanoma, whereas in other situations, such as the prevention of bowel tumors, the risk of more severe autoimmune diseases would demand a careful approach. Experimental studies should address this issue in detail.

Another limitation to be carefully weighed is the constraint imposed by the polymorphism of MHC glycoproteins and the repertoire of peptides presented. Different peptides would need to be prepared to fit in the polymorphic peptide-binding clefts of the many MHC class I and II glycoproteins. It is predictable that certain TAAs will have a restricted usage, and a few individuals will not be easily vaccinated.

Elicitation of a "surgical" immune response ablating only cells that express a specific antigen is probably impossible. This does not mean, however, that individualized vaccines are a strict necessity. Vaccines have to be reprocessed by the immune system of the host. Therefore, in many instances, the presence of inappropriate antigens, for example, allogeneic MHC molecules, could result in the establishment of a polyclonal T-cell activator that would favor and not hamper the induction of a restricted, peptide-specific immune response (43, 44, 55).

[4] A. De Stefani, G. Forni, R. Ragona, G. P. Cavallo, M. Bussi, A. Usai, F. Badellino, and G. Cortesina. Improved survival with perilymphatic IL-2 in resectable squamous cell carcinomas of the oral cavity and oropharynx, submitted for publication.

Conclusions

The cardinal prerequisite of preventive medicine is the Hippocratic "do no harm": *"primum non nocere"* (56). Prolonged nonspecific and specific immune stimulation of persons at risk and the general population is indeed not free from uncertainty, although identification of the steps of tumor progression most susceptible to the immune mechanisms elicited could drastically reduce the stimulation period (42).

However, as stressed in a recent report on cancer chemoprevention (56), failure to intervene when a disease as diffuse and dramatic as cancer can be prevented can also be viewed as harmful. The idea that it is not appropriate to treat healthy persons with cytokines or with antitumor vaccines because of the risks involved will hopefully be shown to be a misconception. An equal or even higher risk of inducing autoimmune complications is associated with many antimicrobial vaccines. Fortunately, they came into use before this risk was perceived. Had it been otherwise, their employment would have been much more strongly opposed, and many more persons would have died.

In conclusion, immunoprevention of cancer seems a distant but plausible prospect. Experimental elucidation of its critical issues could provide essential information for its application in humans. Prevention itself would provide a fresh and perhaps conclusive way of winning the long-lasting war against cancer. Manipulation of the immune response to prevent cancer could soon lead to the realization of a notion that has deep roots in the history of immunology (57).

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